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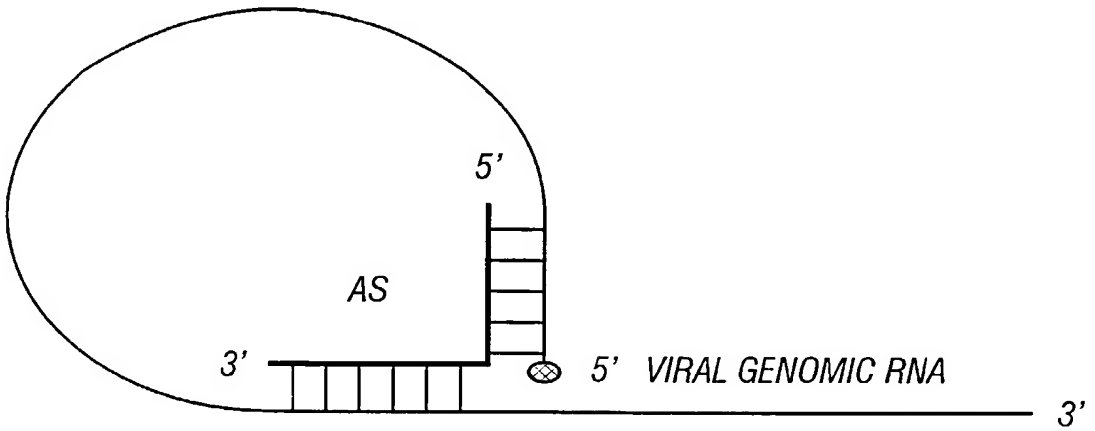

(57) Abstract: The present invention describes antisense nucleic acids compositions comprising sequences complementary to a first and second region in a target nucleic acid. The antisense sequences are designed to hybridize to complementary nucleic acid target regions in a target RNA, and inhibit translation, processing, transport, or binding by proteins or riboproteins. The first and the second target regions may be contiguous or non-contiguous in the target nucleic acid. Target regions include, but are not limited to, AUG, 5' non-translated sequences, translation initiation factor binding sites, ribosome subunit binding sites, Shine Dalgarno sequence, 3' nontranslated sequences, poly-A tail, 3' cleavage site, coding region, intron, intron branch site, intron/exon junction, or splice sequence. Target nucleic acid may be viral, bacterial, animal, human, and plant. Methods for using these antisense compositions include inhibiting translation of an RNA and treating diseases in a cell, an animal, a plant or a human. Pharmaceutical antisense compositions are also described.

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DESCRIPTION

ANTISENSE COMPOSITIONS AND METHODS

BACKGROUND OF THE INVENTION

This application claims priority to provisional U.S. patent application Serial No. 60/175,141 filed January 7, 2000.

The government owns rights in the present invention pursuant to USDA grant number 94-39210-0371.

1. Field of the Invention

The present invention relates generally to the fields of biochemistry and cell biology. More particularly, it concerns antisense compositions and methods.

2. Description of Related Art

The term "antisense" originally referred to an oligonucleotide sequence designed to complement a pre-mRNA or mRNA, both of which are "sense" molecules, so that the ultimate translation of that RNA would be inhibited. Theoretically, when the two molecules were in close proximity, they would hybridize to each other and the target RNA would therefore no longer be accessible to translation initiation factors and ribosomes. In effect, the gene coding for the target RNA would be "turned off." This definition of antisense now includes oligonucleotides ("ON"; short RNA molecules) and oligodeoxynucleotides ("ODN"; short, single stranded DNA molecules) that bind double stranded DNA or areas of double stranded secondary structure in RNA to form triplexes. Indeed, the current definition of antisense covers the entire range of ON/ODN/nucleic acid interaction.

In the mid 1970s, some initial research was done using antisense compounds, but the idea of using complementary nucleic acid sequences to affect DNA or RNA function was not seriously pursued until the mid to late 1980s. At that time two

developments made widespread antisense research possible: (1) protein and DNA sequencing technology and (2) nucleic acid synthesizing technology. Now the sequences of target RNAs could be determined and antisense molecules against these RNAs could be synthesized. Since then, research using antisense strategies has greatly increased.

Historically antisense has been used in two main areas of research: loss-of-function studies and therapeutic use. In loss-of-function studies, a gene or its mRNA is "turned off" by complementary antisense and the resulting phenotype noted. RNA and DNA antisense have been used to both control and study the functions of many diverse genes in a variety of prokaryotic and eukaryotic organisms (Green *et al.* 1986; Mol *et al.* 1990; Murray 1992). Technology involving the development of antisense therapeutics is also showing rapid growth. In antisense therapeutics, disease-associated genes or their RNA transcripts are targeted by antisense molecules designed to inhibit their expression (Brysch and Schlingensiepen 1994; Crooke 1998; Lavrovsky *et al.* 1997; Uhlmann and Peyman 1990). Some of the primary targets for therapeutic antisense design have been proto-oncogenes, such as c-*myc*, N-*myc*, c-*myb*, c-*fos*, N-*ras*, c-H-*ras*, BCL-2, c-*raf*-1, *cdc*-2 and c-*mos* (Fabbro *et al.* 1998) and some of the DNA and RNA viruses (Cohen 1991; Cowsert *et al.* 1993; Field 1998). However, antisense therapeutic agents have also been tested or are currently in clinical trials against such diverse conditions as rheumatoid arthritis (Nietfeld *et al.* 1994), restenosis after coronary angioplasty (Epstein *et al.* 1992), IgE-induced allergic reactions (Hall and Brostoff 1992), renal transplant rejection (Shanahan Jr. 1998), Crohn's disease (Yacyshyn *et al.* 1997), and psoriasis (Shanahan Jr. 1998).

Antiviral Antisense

As of 1997, there were 18 non-antisense antiviral drugs approved for internal use (Hutcherson 1998). Half of these are specific for the human immunodeficiency virus (HIV), inhibiting either the viral reverse transcriptase or the viral protease. Only one of the 18 drugs, ribavirin (ICN), is a broad-spectrum antiviral. The remaining drugs are specific for influenza virus, herpes simplex virus, cytomegalovirus or varicella-zoster virus. In order to find a single effective antiviral drug, hundreds, and sometimes thousands, of compounds must be screened because the drug design

protocols are usually of a random, not rational, nature (De Clerq 1991). Many of these drugs are also cytotoxic to a certain extent and have short half-lives *in vivo* (Fabbro *et al.* 1998). Therefore, the development of antiviral antisense drugs is particularly attractive.

Antiviral antisense drugs have the potential to be quite specific for their viral targets without interfering with other nontarget nucleic acids within the cell. Theoretically, therefore, these drugs should be less cytotoxic. In addition, the design of multiple antisense molecules targeting various sequences within a single virus would permit rapid drug rotation in response to the development of resistance by that virus. In fact, there are indications that such antiviral antisense "cocktails" may be necessary when targeting viral populations having a high degree of genetic variability (Bull *et al.* 1998). However, Bull *et al.* also suggest that the solution to this problem may involve simply targeting viral sequences that are relatively unchanging within that viral population.

Effective antisense design is still routinely a trial-and-error process with complementarity to the nucleic acid target being only one of the design considerations. Other factors that have been reported as affecting the effectiveness of an antisense design include nonspecific interactions of the antisense with nontarget nucleic acids and nucleic acid-binding proteins, as well as the sequence context of antisense:target hybridization. This last factor includes not only the actual linear base sequence, but also the secondary and tertiary structures formed by both the antisense and the target. However, even with these limitations, antisense drug design is a more rational process than most traditional drug design protocols. In addition, it is relatively easy and inexpensive to design and test entire antisense libraries targeting a particular viral sequence.

Antisense Design and Test Systems

Much of the current antisense research has focused on designing more stable, noncytotoxic ODNs for therapeutic use that are specific for the target nucleic acids. ODNs have been the most frequent form of antisense molecule used because of the greater intracellular stability of antisense DNA as compared to antisense RNA. Most

ODNs in use today are relatively short (12-25 nucleotides) and are chemically modified to increase their resistance to intracellular nucleases and to increase their affinity for the target molecules (Uhlmann and Peyman 1990). Unfortunately, these chemical modifications are often accompanied by an increase in cytotoxity, which limits the use of such ODNs *in vivo*. However, one group of ODNs, the phosphorothioate ODNs, has exhibited minimal cytotoxicity and a high level of intracellular stability (Bennett 1998). In these ODNs, one of the oxygen atoms of the phosphate group normally found in the nucleic acid backbone is replaced with a sulfur atom (Cohen 1993). Although the sulfur increases the nuclease resistance of this ODN, the intracellular half-life is still only 12 to 24 hours (Crooke *et al.* 1995). As of 1996, nineteen phosphorothioate antisense drugs had been allowed into clinical trials (Dean *et al.* 1996).

Chemically unmodified antisense has also been utilized against a variety of RNAs with varying degrees of success (Weiss *et al.* 1999). One way of producing this kind of antisense is by reverse transcribing a complementary DNA (cDNA) from a target mRNA and then cloning this cDNA into an expression vector in an antisense orientation. The cloned nucleotide sequence is therefore reversed as compared with the normal sequence and RNA transcripts produced from this cloned sequence are able to hybridize with the target mRNA. The expression vector is then either transfected or injected into the target cells. These vectors are transient, but remain within the cell long enough for antisense RNA transcription to occur. Such expression vectors are quite convenient for rapidly screening a variety of antisense molecules to see which ones will affect translation of the target mRNAs.

Antisense in Plants

The use of antisense in plants has generally been geared more toward regulating and/or investigating plant gene expression rather than toward controlling viral infections. This was due primarily to the early development of relatively effective viral control and prevention measures.

Historically, crop viruses have been partially controlled by crop management practices such as crop rotation, insect/nematode/fungal vector control, the planting of

virus-free seed, and the development of virus-resistant plant varieties by selective breeding. Unfortunately, these control measures are limited in their ability to prevent virus infections. Another virus control measure is cross protection. Cross protection is a process whereby a strain of the plant virus that produces only mild symptoms in the plant is inoculated onto the host plant. This mild virus infection protects the plant from infection by a more virulent strain of the virus (Fulton 1986 from PV). Cross protection does work although crop yields are often slightly reduced.

In 1986, Abel *et al.* transformed tobacco with the coat protein gene of TMV, a Tobamovirus, in a "sense" orientation so that a normal TMV coat protein mRNA was now expressed in the transgenic tobacco plant (Abel *et al.* 1986). Tobacco is normally very susceptible to TMV infection, but the new transgenic plant was not. In addition, these plants were also resistant to certain of the Tobamoviruses closely related to TMV (Nejidat and Beachy 1990). Since that time, coat protein-mediated resistance (CPMP) has been used successfully against a variety of plant viruses. Other viral genes, such as replicase and movement protein genes, have also been used as host plant transgenes but with varying degrees of success (Beachy 1997).

Much of the antisense RNA designed today is chemically modified to increase its stability in a cellular environment and to increase its affinity for the target RNA. This kind of modification is typically seen in exogenously introduced antisense RNA but endogenously produced antisense RNA (such as that produced by a plant containing an antisense transgene) cannot be modified in this way. The following discussion deals with some design problems common to both types of antisense but the focus will be on ways to optimize the design of endogenous antisense.

There are several potential problems that must be dealt with in designing a successful antisense molecule. One consideration must be the stability of the antisense RNA in the host cell environment. Normal cellular mRNAs have variable degrees of stability within the cytoplasm, depending upon the presence of a 5' cap (mentioned above), both 5' and 3' UTR regulatory elements, and a poly(A) tail of variable length. Another design consideration must be the secondary and tertiary structures formed by both the target RNA and the antisense RNA. Much of the past

antisense research has focused on predicting areas of linearity in the target RNA and designing relatively short, linear RNAs complementary to the target sequence. Unfortunately, it is often difficult to determine where a linear sequence might be within the target RNA. As previously mentioned, in many of the naturally-occurring antisense systems, the antisense and target RNAs are not predicted to be linear at all. Indeed, secondary structures seem to be required for initial binding between the two RNAs (Hjalt and Wagner 1995; Simons 1993). A high degree of complementarity, however, is important.

Antisense length also seems to be important. Specificity for a particular target RNA requires an antisense length of at least 11 to 15 nucleotides (Helene and Toulme 1990). Below that length, the antisense tends to bind nonspecifically to a variety of targets. As antisense length increases, the affinity of the antisense for its target also potentially increases because of the increased number of hydrogen bonds possible between the two molecules (Mirabelli and Crooke 1993). There are dangers though in increasing antisense length -- the antisense RNA has more opportunity to partially bind to nontarget RNAs (therefore decreasing its specificity for the target) and it may develop a higher degree of secondary structure (Ecker 1993) which may affect its ability to stably bind to the target. Affinity is determined not only by antisense length but also by the GC versus AU content of the duplex. A high GC content allows the antisense to bind strongly to nontarget RNAs even if there is a partial mismatch between the two molecules, whereas an antisense RNA with high AU content may not bind strongly even to its target sequence. Brysch (1994) therefore suggests that the best specificity and affinity is found in antisense RNAs containing an even distribution of AUs and GCs.

Ecker *et al.* (1992) have developed some optimization rules for antisense design. They include the following: (1) avoid targeting long stems but instead target single stranded areas, short stems, and bulges; (2) avoid antisense designs that contain extremely stable secondary or tertiary structures; (3) choose antisense lengths that allow all bases to bind the target. Again, the ribosome's ability to unwind double stranded structures within its template RNA must be considered. In competition studies done by Lawson *et al* (1989), eIF-4F was able to remove oligonucleotides

complementary to the first 15 nucleotides of capped mRNA. Therefore, it might be more efficient to design antisense that prevents the initial binding of the ribosomal subunits rather than to attempt to design antisense with a high enough affinity for the target that the ribosome could not remove it.

The present invention concerns novel antisense compositions that overcome many of these problems and are more efficient at inhibitng expression of targetted genes.

SUMMARY OF THE INVENTION

This invention is based on antisense nucleic acid compositions comprising sequences complementary to a first region in a target nucleic acid and to a second region in a target nucleic acid. The antisense sequences are designed to hybridize to complementary nucleic acid target regions in a target nucleic acid, and inhibit translation, processing, transport, or binding by proteins or riboproteins. The sequences of the antisence nucleic acid compositions may be contiguous or non-contiguous. The first and second regions of the target nucleic acid are not contiguous. The invention encompasses antisense nucleic acid compositions and methods for their use.

In one embodiment, the invention provides an antisence nucleic acid comprising sequences complementary to a first region in a target nucleic acid and a second region in the target nucleic acid. In another embodiment, the invention provides an antisence nucleic acid comprising a first sequence complementary to a first region in a target nucleic acid and a second sequence complementary to a second region in the target nucleic acid. In other embodiments, the invention comprises method of contacting a target nucleic acid with the antisence nucleic acids described above.

In some embodiments, one or both of the first or second sequences of the antisense nucleic acid may be complementary to regions of the target nucleic acid that comprise, for example, a 5' non-translated region, an AUG, a translation initiation

factor binding sequence, a ribosome subunit binding sequence, a Shine Dalgarno sequence, a 3' non-translated sequence, a poly-addition site, a 3' mRNA cleavage site, a coding region, or an intron, intron branch, intron/exon junction, or a splice sequence. In one embodiment of the invention, the target nucleic acid encodes, for example, one or more oncogenes, angiogenic genes, tumor suppressors, inducers of apoptosis, enzymes, transcription factor regulators, cell cycle regulators, viral sequences and bacterial sequences. In still further embodiments of the invention, the antisense nucleic acid may be about 20, 25, 30, 40, 50, 100, 200, 500, 1000, 1500, 2000, or about 4000 bases or longer. The antisense nucleic acid may be comprised in an expression vector.

In some embodiments the target nucleic acid is mRNA. In other embodiments the invention encompasses a method of inhibiting transcription of mRNA. In other embodiments, the invention includes methods of treating a disease state that is associated with expression of a selected gene product, wherein the target nucleic acid is an mRNA encoding the selected gene product.

In further embodiments, the target nucleic acid is in a cell. The type of such cell may be, for example, an animal cell, a plant cell, and a human cell.

In some embodiments, the invention provides a method for protecting a cell from pathogen attack comprising contacting a nucleic acid of said pathogen with an antisense nucleic acid comprising sequences complementary to two regions in a target nucleic acid, wherein the target nucleic acid is a pathogen nucleic acid. In one embodiment, the pathogen is a virus. The cell may be a plant or animal cell and the target nucleic acid may be mRNA. In one embodiment, the protecting comprises inhibiting viral replication. The antisense nucleic acid may be comprised in an expression vector.

In embodiments of the present invention in which the first and second sequences are not contiguous in the antisense nucleic acid, the first sequence and the second sequence may be linked by a linker. The linker may comprise, for example, a

nucleic acid segment, or non-nucleic acid such as an organic or inorganic chemical group.

In another embodiment, the invention provides a method for screening for genes of a given function using the loop-inducing antisense format as a more predictably effective means to inhibit gene expression.

In still another embodiment, the invention comprises a pharmaceutical composition comprising an antisense nucleic acid comprising sequences complementary to two regions in a target nucleic acid, wherein the regions are not contiguous in the target nucleic acid. In another embodiment of the invention, the pharmaceutical composition is dispersed in a pharmacologically acceptable buffer, diluent or excipient. In the pharmaceutical composition, the antisense nucleic acid may be comprised in an expression vector, may further be of any type contemplated by the invention.

In still yet another embodiment, the invention provides a cell comprising an antisense nucleic acid in accordance with the invention. The cell may comprise a eukaryotic cell, including an animal, human or plant cell. Another embodiment of the invention includes a plant or animal comprising such a cell.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A, FIG. 1B and FIG. 1C. General replication scheme used by positive sense single stranded RNA viruses. Also note the double stranded replicative forms of this RNA virus (● = viral replicase).

FIG. 2. Construction of loop-inducing antisense targeting TMV's genomic and negative sense RNAs.

FIG. 3. Theoretical mechanism of loop-inducing antisense action (AS = antisense).

FIG. 4A and FIG. 4B. Construction of pPZP-AL plasmids.

FIG. 5. TFP transgene RNA expression levels. All plants gave a positive value except nontransgenic plants, which had density values of zero.

FIG. 6. TFN transgene RNA expression levels. All plants gave a positive value except nontransgenic plants, which had density values of zero.

FIG. 7. Development of systemic TMV infection in TFP plants. All plants shown in FIG. 5 and FIG. 6 were initially screened for resistance to TMV. The most resistant plants were selected for further screening, as seen here and in subsequent figures.

FIG. 8. Development of systemic TMV infection in TFN plants.

FIG. 9. Development of systemic TMV infection in TQP plants.

FIG. 10. Development of systemic TMV infection in TQN plants.

FIG. 11. Development of systemic TMV infection in T_0 plants.

FIG. 12. Time of first appearance of lesions. Bars from left to right are for the following plants in each series: (GFP (55); GFN (31);GQP (44); GQN (24);Gzero (7)). Numbers in () indicate the number of individual plants used to get the average value.

FIG. 13. Total lesion number for transgenic *Nicotiana tabacum* cv. *Xanthi NN*. Bars from left to right are for the following plants in each series: (GFP (41); GFN (44); GQP (72); GQN (10). Numbers in () indicate the number of individual plants used to get the average value.

FIG. 14. Average lesion diameters for GFP plants

FIG. 15. Average lesion diameters for transgenic *Nicotiana tabacum* cv. *Xanthi NN*.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention overcomes deficiencies in the prior art by describing antisense nucleic acids compositions comprising a first and a second domain. The domains are designed to hybridize to complementary nucleic acid target domains in a target RNA, and inhibit translation, processing, transport, or binding by proteins or riboproteins. The first and the second target domain may be contiguous or non-contiguous in the target RNA. Target domains include, for example, AUG, 5' non-translated sequences, translation initiation factor binding sites, ribosome subunit binding sites, Shine Dalgarno sequence, 3' nontranslated sequences, poly-A tail, 3' cleavage sites, coding regions, introns, intron branch sites, intron/exon junctions, or splice sequences. Target RNA may be viral, bacterial, animal, human, and plant. Methods for using these antisense compositions include inhibiting translation of an RNA and treating diseases in a cell, an animal, a plant or a human. Therapeutic antisense compositions are also described.

The following section outlines existing antisense technology, including antisense transgene construction, viral and non-viral delivery and expression vectors, and antisense oligonucleotides. Descriptions of examples are given for target domains and target genes as well as methods for administering antisense compositions and disease treatment.

I. Antisense

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. The term "complementary" is intended to refer to polynucleotides that are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and either adenine paired with thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine *et al.* in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense construct polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription, translation, or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences that are completely complementary will be sequences that are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology are also contemplated. For example, an antisense construct that has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see below) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

A. Antisense Transgenes

Within certain embodiments expression vectors used in therapeutic applications. Expression requires that appropriate signals be provided in the vectors, which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells are also defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a polynucleotide coding for a gene product in which part or all of the polynucleotide encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the polynucleotide encoding a gene of interest.

In preferred embodiments, the polynucleotide encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the polynucleotide to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a polynucleotide sequence of interest is not believed to be important, so long as it is capable of directing the expression of the polynucleotide in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. For example in the case where expression of a transgene, or transgenes when a multicistronic vector is utilized, is toxic to the cells in which the vector is produced in, it may be desirable to prohibit or reduce expression of one or more of the transgenes. Examples of transgenes that may be toxic to the producer cell line are pro-apoptotic and cytokine genes. Several inducible promoter systems are available for production of viral vectors where the transgene product may be toxic.

The Ecdysone-Inducible Mammalian Expression System (Invitrogen, Carlsbad, CA) is one such system. This system is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility. The system is based on the heterodimeric ecdysone receptor of *Drosophila*, and when ecdysone or an analog such as muristerone A binds to the receptor, the receptor activates a promoter to turn on expression of the downstream transgene high levels of mRNA transcripts are attained. In this system, both monomers of the heterodimeric receptor are constitutively expressed from one vector, whereas the ecdysone-responsive promoter which drives expression of the gene of interest is on another plasmid. Engineering of this type of system into the gene transfer vector of interest would therefore be useful. Cotransfection of plasmids containing the gene of interest and the receptor monomers in the producer cell line would then allow for the production of the gene transfer vector without expression of

a potentially toxic transgene. At the appropriate time, expression of the transgene could be activated with ecdysone or muristeron A.

Another inducible system that would be useful is the Tet-Off™ or Tet-On™ system (Clontech, Palo Alto, CA) originally developed by Gossen and Bujard (Gossen and Bujard, 1992; Gossen *et al.,* 1995). This system also allows high levels of gene expression to be regulated in response to tetracycline or tetracycline derivatives such as doxycycline. In the Tet-On™ system, gene expression is turned on in the presence of doxycycline, whereas in the Tet-Off™ system, gene expression is turned on in the absence of doxycycline. These systems are based on two regulatory elements derived from the tetracycline resistance operon of *E. coli.* The gene of interest is cloned into a plasmid behind a promoter that has tetracycline-responsive elements present in it. A second plasmid contains a regulatory element called the tetracycline-controlled transactivator, which is composed, in the Tet-Off™ system, of the VP16 domain from the herpes simplex virus and the wild-type tertracycline repressor. Thus in the absence of doxycycline, transcription is constitutively on. In the Tet-On™ system, the tetracycline repressor is not wild type and in the presence of doxycycline activates transcription. For gene therapy vector production, the Tet-Off™ system would be preferable so that the producer cells could be grown in the presence of tetracycline or doxycycline and prevent expression of a potentially toxic transgene, but when the vector is introduced to the patient, the gene expression would be constitutively on.

An inducible system particularly useful with the present invention is a radiation-inducible system. Ionizing radiation-inducible promoters include a CArG domain of an *Egr-1* promoter, a *los* promoter, a c-jun promoter, and a TNF-α promoter, which can be operatively linked to a protein expression region. In this regard, U.S. Patent 5,612,318, dealing with induction of expression from these promoters, is specifically incorporated by reference.

In some circumstances, it may be desirable to regulate expression of a transgene in a gene therapy vector. For example, different viral promoters with varying strengths of activity may be utilized depending on the level of expression

desired. In mammalian cells, the CMV immediate early promoter if often used to provide strong transcriptional activation. Modified versions of the CMV promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. When expression of a transgene in hematopoetic cells is desired, retroviral promoters such as the LTRs from MLV or MMTV are often used. Other viral promoters that may be used depending on the desired effect include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters such as from the E1A, E2A, or MLP region, AAV LTR, cauliflower mosaic virus, HSV-TK, and avian sarcoma virus.

Similarly tissue specific promoters may be used to effect transcription in specific tissues or cells so as to reduce potential toxicity or undesirable effects to non-targeted tissues. For example, promoters such as the PSA, probasin, prostatic acid phosphatase or prostate-specific glandular kallikrein (hK2) may be used to target gene expression in the prostate.

In certain indications, it may be desirable to activate transcription at specific times after administration of the gene therapy vector. This may be done with such promoters as those that are hormone or cytokine regulatable. For example in gene therapy applications where the indication is a gonadal tissue where specific steroids are produced or routed to, use of androgen or estrogen regulated promoters may be advantageous. Such promoters that are hormone regulatable include MMTV, MT-1, ecdysone and RuBisco. Other hormone regulated promoters such as those responsive to thyroid, pituitary and adrenal hormones are expected to be useful in the present invention. Cytokine and inflammatory protein responsive promoters that could be used include K and T Kininogen (Kageyama *et al.,* 1987), c-fos, TNF-alpha, C-reactive protein (Arcone *et al.,* 1988), haptoglobin (Oliviero *et al.,* 1987), serum amyloid A2, C/EBP alpha, IL-1, IL-6 (Poli and Cortese, 1989), Complement C3 (Wilson *et al.,* 1990), IL-8, alpha-1 acid glycoprotein (Prowse and Baumann, 1988), alpha-1 antitypsin, lipoprotein lipase (Zechner *et al.,* 1988), angiotensinogen (Ron *et al.,* 1991), fibrinogen, c-jun (inducible by phorbol esters, TNF-alpha, UV radiation, retinoic acid, and hydrogen peroxide), collagenase (induced by phorbol esters and retinoic acid), metallothionein (heavy metal and glucocorticoid inducible),

Stromelysin (inducible by phorbol ester, interleukin-1 and EGF), alpha-2 macroglobulin and alpha-1 antichymotrypsin.

Tumor specific promoters such as osteocalcin, hypoxia-responsive element (HRE), MAGE-4, CEA, alpha-fetoprotein, GRP78/BiP and tyrosinase may also be used to regulate gene expression in tumor cells. Other promoters that could be used according to the present invention include Lac-regulatable, chemotherapy inducible (*e.g.* MDR), and heat (hyperthermia) inducible promoters, radiation-inducible (*e.g.*, EGR (Joki *et al.,* 1995)), Alpha-inhibin, RNA pol III tRNA met and other amino acid promoters, U1 snRNA (Bartlett *et al.,* 1996), MC-1, PGK, β-actin and α-globin. Many other promoters that may be useful are listed in Walther and Stein (1996).

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are frequently overlapping and contiguous, often seeming to have a very similar modular organization.

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

B. Viral Expression Vectors

There are a number of ways to introduce expression vectors into cells. In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kb of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial

cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5′-tripartite leader (TPL) sequence which makes them preferred mRNAs for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.,* 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package

approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.,* 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Recently, Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 hours. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 hours.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of

any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.,* 1963; Top *et al.,* 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.,* 1991; Gomez-Foix *et al.,* 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.,* 1990; Rich *et al.,* 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.,* 1991; Rosenfeld *et al.,* 1992), muscle injection (Ragot *et al.,* 1993),

peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.,* 1993).

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a polynucleotide encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.,* 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.,* 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.,* 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the

chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.,* 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.,* 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.,* 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.,* 1988; Hersdorffer *et al.,* 1990).

Lentiviruses can also be used as vectors in the present application. In addition to the long-term expression of the transgene provided by all retroviral vectors, lentiviruses present the opportunity to transduce nondividing cells and potentially achieve regulated expression. The development of lentiviral vectors requires the design of transfer vectors to ferry the transgene with efficient encapsidation of the transgene RNA and with full expression capability, and of a packaging vector to provide packaging machinery *in trans* but without helper virus production. For both vectors, a knowledge of packaging signal is required-the signal to be included in the transfer vector but excluded from the packaging vector. Exemplary human lentiviruses are human immunodeficiency virus type 1 and type 2 (HIV-1 and HIV-2).

HIV-2 is likely better suited for gene transfer than HIV-1 as it is less pathogenic and thus safer during design and production; its desirable nuclear import and undesirable cell-cycle arrest functions are segregated on two separate genes (Arya *et al.*, 1998; Blomer *et al.*, 1997).

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription.

The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-structural proteins apparently are derived from the longer of the transcripts, and three virion proteins all arise from the smallest transcript.

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of the helpers is adenovirus, and many "early" functions for this virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

The terminal repeats of an AAV vector can be obtained by restriction endonuclease digestion of AAV or a plasmid such as psub201, which contains a modified AAV genome (Samulski *et al.* 1987), or by other methods known to the skilled artisan, including but not limited to chemical or enzymatic synthesis of the terminal repeats based upon the published sequence of AAV. The ordinarily skilled artisan can determine, by well-known methods such as deletion analysis, the

minimum sequence or part of the AAV ITRs which is required to allow function, *i.e.* stable and site-specific integration. The ordinarily skilled artisan also can determine which minor modifications of the sequence can be tolerated while maintaining the ability of the terminal repeats to direct stable, site-specific integration.

AAV-based vectors have proven to be safe and effective vehicle for gene delivery *in vitro,* and these vectors are now being developed and tested in pre-clinical and clinical stages for a wide range of applications in potential gene therapy, both *ex vivo and in vivo.* However, wide variations in AAV transduction efficiency in different cells and tissues *in vitro* as well as *in vivo* has been repeatedly observed (Ponnazhagan *et al.*, 1997b; 1997c; 1997d; 1997d) *et al.* (Carter and Flotte, 1996 ; Chatterjee *et al.*, 1995; Ferrari *et al.*, 1996; Fisher *et al.*, 1996; Flotte *et al.*, 1993; Goodman *et al.*, 1994; Kaplitt *et al.*, 1994; 1996, Kessler *et al.*, 1996; Koeberl *et al.*, 1997; Mizukami *et al.*, 1996; Xiao *et al.*, 1996).

AAV-mediated efficient gene transfer and expression in the lung has led to clinical trials for the treatment of cystic fibrosis (Carter and Flotte, 1996; Flotte *et al.*, 1993). Similarly, the prospects for treatment of muscular dystrophy by AAV-mediated gene delivery of the dystrophin gene to skeletal muscle, of Parkinson's disease by tyrosine hydroxylase gene delivery to the brain, of hemophilia B by Factor IX gene delivery to the liver, and potentially of myocardial infarction by vascular endothelial growth factor gene to the heart, appear promising since AAV-mediated transgene expression in these organs has recently been shown to be highly efficient (Fisher *et al.*, 1996; Flotte *et al.*, 1993; Kaplitt *et al.*, 1994; 1996; Koeberl *et al.*, 1997; McCown *et al.*, 1996; Ping *et al.*, 1996; Xiao *et al.*, 1996).

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988; Horwich *et al.,* 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.,* 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* (1991) recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was co-transfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.,* 1991).

C. Non-viral Delivery of Expression Vectors

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.,* 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In yet another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver

and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

In still another embodiment of the invention, transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.,* 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.,* 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.,* 1990; Zelenin *et al.,* 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e., ex vivo* treatment. Again, DNA encoding a particular gene may be delivered via this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.,* (1980) demonstrated the feasibility of

liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.,* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.,* 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.,* 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. Since these expression constructs have been successfully employed in transfer and expression of polynucleotides *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a polynucleotide encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.,* 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.,* 1993; Perales *et al.,* 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a polynucleotide encoding a particular gene also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of a polynucleotide encoding a gene in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a polynucleotide into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

D. Antisense Oligonucleotides

Antisense oligodeoxynucleotides (AS-ODNs) are single-stranded, short sequences of DNA (Cohen, 1989; De Mesmaeker *et al.*, 1995) that are complementary to specific messenger RNA (mRNA). Since AS-ODNs hybridize with the mRNA, they prevent the targeted mRNA from expressing its polypeptide product in the cell.

The oligonucleotides (or "ODNs" or "polynucleotides" or "oligos" or "oligomers" or "n-mers") of the present invention are preferably deoxyoligonucleotides (*i.e.* DNAs), or derivatives thereof; ribo-oligonucleotides (*i.e.* RNAs) or derivatives thereof; or peptide nucleic acids (PNAs) or derivatives thereof. The oligonucleotides may also comprise phosphorothioate antisense oligonucleotides.

The term "substantially complementary," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, an oligonucleotide sequence, is substantially complementary to the sequence, and thus will specifically bind to a portion of an mRNA. As such, typically the sequences will be highly complementary to the mRNA "target" sequence, and will have no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches throughout the sequence. In many instances, it may be desirable for the sequences to be exact matches, *i.e.* be completely complementary to the sequence to which the oligonucleotide specifically binds, and therefore have zero mismatches along the complementary stretch. As such, highly complementary sequences will typically bind quite specifically to the target sequence region of the mRNA and will therefore be highly efficient in reducing, and/or even inhibiting the translation of the target mRNA sequence into polypeptide product.

Substantially complementary oligonucleotide sequences will be greater than about 80 percent complementary (or '% exact-match') to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary oligonucleotide sequences for use in the practice of the invention, and in such instances, the oligonucleotide sequences will be greater than about 90 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and may in certain embodiments be greater than about 95 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and even up to and including 96%, 97%, 98%, 99%, and even 100% exact match complementary to the target mRNA to which the designed oligonucleotide specifically binds.

Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of

Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

"DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct. Preferably, the DNA sequences are in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences could also be used. Sequences of non-translated DNA may be present 5′ or 3′ from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

"RNA sequence" refers to an RNA polymer, in the form of a separate fragment or as a component of a larger RNA construct, such as a messenger RNA (mRNA). Preferably, the RNA sequences are in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector, or alternatively, by chemically synthesizing the RNA molecule completely or partially *in vitro*.

"Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides, ribonucleotides, or peptide-nucleic acid sequences that may be assembled from smaller fragments, isolated from larger fragments, or chemically synthesized *de novo* or partially synthesized by combining shorter oligonucleotide linkers, or from a series of oligonucleotides, to provide a sequence which is capable of specifically binding to an mRNA molecule and acting as an antisense construct to alter, reduce, or inhibit the

transcription of the message into polypeptide, and thus, ultimately affect the concentration, amount, or activity of the final gene product *in situ*, *in vitro*, or *in vivo*.

The targeting of antisense oligonucleotides to bind mRNA is one mechanism to shut down protein synthesis. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829, each specifically incorporated herein by reference in its entirety). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal $GABA_A$ receptor and human EGF (Jaskulski *et al.*, 1988; Vasanthakumar and Ahmed, 1989; Peris *et al.*, 1998; U. S. Patent 5,801,154; U. S. Patent 5,789,573; U. S. Patent 5,718,709 and U. S. Patent 5,610,288, each specifically incorporated herein by reference in its entirety). Antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683, each specifically incorporated herein by reference in its entirety).

The oligonucleotide compounds of the invention bind to the messenger RNA coding for human ACE thereby inhibiting expression of the protein. In the specification and claims, the letters, A, G, C, T, and U respectively indicate nucleotides in which the nucleoside is Adenosine (Ade), Guanosine (Gua), Cytidine (Cyt), Thymidine (Thy), and Uridine (Ura). As used in the specification and claims, compounds that are antisense to the DNA or mRNA sense strand are compounds which have a nucleoside sequence complementary to the sense strand. Table 1 shows the four possible sense strand nucleosides and their complements present in an antisense compound.

TABLE 1

Sense	Antisense
Ade	Thy
Gua	Cyt
Cyt	Gua
Thy	Ade
Ura	Ade

It will be understood by those skilled in the art that the present invention broadly includes oligonucleotide compounds which are capable of binding to the sense mRNA. Thus, the invention includes compounds which are not strictly antisense: the compounds may have some non-complementary bases provided such compound have sufficient binding affinity for mRNA to inhibit expression.

The antisense compounds of the present invention may also differ from native DNA in that some or all of the phosphates in the nucleotides are replaced by phosphorothioates (X=S) or methylphosphonates ($X=CH_3$) or other C_{1-4} alkylphosphonates. The compounds may be further differentiated from native DNA by replacing one or both of the free hydroxy groups of the antisense molecule with C_{1-4} alkoxy groups ($R=C_{1-4}$ alkoxy). As used herein, C_{1-4} alkyl means a branched or unbranched hydrocarbon having 1 to 4 carbon-atoms.

Antisense compounds also may be substituted at the 3′ and/or 5′ ends by a substituted acridine derivative. As used herein, "substituted acridine" means any acridine derivative capable of intercalating nucleotide strands such as DNA. Preferred substituted acridines are 2-methoxy-6-chloro-9-pentylaminoacridine, N-(6-chloro-2-methoxyacridinyl) -O-methoxydiisopropylaminophosphinyl-3-aminopropanol, and N-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-5-aminopentanol. Other suitable acridine derivatives are readily apparent to persons skilled in the art. Additionally, as used herein "P(0) (0) -substituted acridine" means a phosphate covalently linked to a substitute acridine.

The oligonucleotide antisense compounds have at least 9 to about 35 or so nucleotides in length. As used herein, the term "nucleotides" includes nucleotides in which the phosphate moiety is replaced by phosphorothioate or alkylphosphonate and the nucleotides may be substituted by substituted acridines. Preferred oligonucleotide antisense compounds have at least 9 to about 25 nucleotides, while more preferred compounds have at least 9 to about 15 or so nucleotides. Compounds having fewer than 9 nucleotides are less desirable because they generally have less specificity and compounds having greater than about 35 nucleotides are less desirable because their physical size and charge will attenuate the crossing of the lipophilic cell membrane. Thus, they are less likely to enter cells.

The reaction scheme involves 1H-tetrazole-catalyzed coupling of phosphoramidites to give phosphate intermediates which are reacted with sulfur in 2,6-lutidine to give phosphate compounds. Oligonucleotide compounds are prepared by treating the phosphate compounds with thiophenoxide (1:2:2 thiophenol/triethylamine/tetra-hydrofuran, room temperature, 1 h). The reaction sequence is repeated until an oligonucleotide compound of the desired length has been prepared. Such compounds are cleaved from the support by treating with ammonium hydroxide at room temperature for 1 h and then are further deprotected by heating at about 50°C overnight to yield compounds. Compounds in which at least one X is oxygen are prepared by substituting I_2-H_2O for the sulfur in 2,6-lutidine.

Antisense oligonucleotide compounds in which at least X is CH_3 or other C_{1-4} alkyl are prepared by the following published procedure: Agarwal and Riftina, 1979. The reaction sequence is conducted on a solid support. The reaction procedure involves phosphorylation of the 3′-hydroxyl group of a 5′-protected nucleoside using methylphosphonoditriazolide as the phosphorylating reagent followed by benzene sulfonyl-catalyzed coupling of the methylphosphonates to yield the methyl phosphonate oligonucleotide. Methylphosphonoditriazolide is prepared *in situ* from equimolar quantities of methylphosphonodichloridate, triethylamine, and triazole. Benzene sulfonyl tetrazole also was prepared *in situ* from pyridine, benzene sulfonic acid and triethylamine. Repeating this reaction sequence followed by cleavage from the support and deprotection yield antisense compounds.

Antisense compounds in which R is P(0) (0)-substituted acridine also are prepared by the following published procedures: Asseline and Thuong, 1989; Stein *et al.*, 1988. These published procedures include synthesis of a nucleoside phosphoramidite-bearing acridine derivative which then is reacted with 2, 2′-dithiodiethanol attached to a support. The elongation chain then is carried out of an automatic solid-phase DNA synthesized as described above. These published procedures also include synthesis of nucleoside phosphoramidite-bearing acridine derivatives by reacting substituted 9-(3-hydroxypropyl) amino acridines with N-ethyldiisopropylamine followed by N,N-diisopropylmethylphosphonanidic chloride. Using an automated DNA synthesizer, antisense compounds in which R is P(0) (0)-substituted acridine are prepared by an extra round of synthesis using the acridinyl phosphoramidites in acetonitrile.

Highly preferred target regions of the mRNA, are those which are at or near the AUG translation initiation codon, and those sequences which were substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations were performed using v.4 of the OLIGO primer analysis software (Rychlik, 1997) and the BLASTN 2.0.5 algorithm software (Altschul *et al.,* 1997).

Nucleotides at either end derived by the same process (using OLIGO and BLAST) are also envisioned by this invention. The effective length of the ODN is from 9-25 nucleotides. The inventors contemplate oligonucleotide compositions in the range of from at least 9 to about 35 or so bases in length are most preferred for the practice of the methods of the invention. In illustrative embodiments, the antisense compounds of the invention differ from native DNA by the modification of the phosphodiester backbone to extend the life of the antisense ODN, in which the phosphate substituents are replaced by phosphorothioates. Likewise, one or both ends of the oligonucleotide may be substituted by one or more acridine derivatives which intercalates nucleotide strands of DNA.

II. Target Domains

A. Contiguous/non-contiguous Target Domains

Target domains in the target nucleic acid are defined as being "non-contiguous" in the primary RNA sequence. Non-contiguous target domains are where the first domain and second domain are separated by at least 1 nucleic acid base. In other embodiments, the first domain and second domain are separated by at least 2 nucleic acid bases, or the first domain and second domain are separated by at least 3 nucleic acid bases, or the first domain and second domain are separated by at least 4 nucleic acid bases, or the first domain and second domain are separated by at least 5 nucleic acid bases, or the first domain and second domain are separated by at least 6 nucleic acid bases, or the first domain and second domain are separated by at least 7 nucleic acid bases, or the first domain and second domain are separated by at least 8 nucleic acid bases, or the first domain and second domain are separated by at least 9 nucleic acid bases, or the first domain and second domain are separated by at least 10 nucleic acid bases, or the first domain and second domain are separated by at least 11 nucleic acid bases, or the first domain and second domain are separated by at least 12 nucleic acid bases, or the first domain and second domain are separated by at least 13 nucleic acid bases, or the first domain and second domain are separated by at least 14 nucleic acid bases, or the first domain and second domain are separated by at least 15 nucleic acid bases, or the first domain and second domain are separated by at least 16 nucleic acid bases, or the first domain and second domain are separated by at least 17 nucleic acid bases, or the first domain and second domain are separated by at least 18 nucleic acid bases, or the first domain and second domain are separated by at least 19 nucleic acid bases, or the first domain and second domain are separated by at least 20 nucleic acid bases, or the first domain and second domain are separated by at least 25 nucleic acid bases, or the first domain and second domain are separated by at least 30 nucleic acid bases, or the first domain and second domain are separated by at least 35 nucleic acid bases, or the first domain and second domain are separated by at least 40 nucleic acid bases, or the first domain and second domain are separated by at least 45 nucleic acid bases, or the first domain and second domain are separated by at least 50 nucleic acid bases, or the first domain and second domain are separated by at least 55 nucleic acid bases, or the first domain and second domain are separated by at least 60 nucleic acid

bases, or the first domain and second domain are separated by at least 65 nucleic acid bases, or the first domain and second domain are separated by at least 70 nucleic acid bases, or the first domain and second domain are separated by at least 75 nucleic acid bases, or the first domain and second domain are separated by at least 80 nucleic acid bases, or the first domain and second domain are separated by at least 85 nucleic acid bases, or the first domain and second domain are separated by at least 90 nucleic acid bases, or the first domain and second domain are separated by at least 95 nucleic acid bases, or the first domain and second domain are separated by at least 100 nucleic acid bases, or the first domain and second domain are separated by at least 110 nucleic acid bases, or the first domain and second domain are separated by at least 120 nucleic acid bases, or the first domain and second domain are separated by at least 130 nucleic acid bases, or the first domain and second domain are separated by at least 140 nucleic acid bases, or the first domain and second domain are separated by at least 150 nucleic acid bases, or the first domain and second domain are separated by at least 160 nucleic acid bases, or the first domain and second domain are separated by at least 170 nucleic acid bases, or the first domain and second domain are separated by at least 180 nucleic acid bases, or the first domain and second domain are separated by at least 190 nucleic acid bases, or the first domain and second domain are separated by at least 200 nucleic acid bases, or the first domain and second domain are separated by at least 250 nucleic acid bases, or the first domain and second domain are separated by at least 300 nucleic acid bases, or the first domain and second domain are separated by at least 350 nucleic acid bases, or the first domain and second domain are separated by at least 400 nucleic acid bases, or the first domain and second domain are separated by at least 450 nucleic acid bases, or the first domain and second domain are separated by at least 500 nucleic acid bases, or the first domain and second domain are separated by at least 600 nucleic acid bases, or the first domain and second domain are separated by at least 700 nucleic acid bases, or the first domain and second domain are separated by at least 800 nucleic acid bases, or the first domain and second domain are separated by at least 900 nucleic acid bases, or the first domain and second domain are separated by at least 1000 nucleic acid bases, or the first domain and second domain are separated by at least 1200 nucleic acid bases, or the first domain and second domain are separated by at least 1400 nucleic acid bases, or the first domain and second domain are separated by at least 1600 nucleic acid bases, or the first domain and second domain are separated by at least 1800 nucleic acid

bases, or the first domain and second domain are separated by at least 2000 nucleic acid bases, or the first domain and second domain are separated by at least 2500 nucleic acid bases, or the first domain and second domain are separated by at least 3000 nucleic acid bases, or the first domain and second domain are separated by at least 3500 nucleic acid bases, or the first domain and second domain are separated by at least 4000 nucleic acid bases, or the first domain and second domain are separated by at least 4500 nucleic acid bases, or the first domain and second domain are separated by at least 5000 nucleic acid bases.

B. Cellular

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected. Other domains include AUG, 5' non-translated, Translation Initiation Factor, Ribosome Subunit Binding, Snine Dalgarno, 3' nontranslated, Poly A, 3' Cleavage site, Coding Region, Intron, etc. The site of attachment of the antisense construct may be less concerned with the functionality of that site than with the specific RNA secondary structure of the site. The induction of loop formation and sequestration of the 5' cap is thought to be most important.

C. Viral

The positive sense single stranded RNA viruses are represented by 27 of the 35 plant virus groups and 6 of the 22 vertebrate virus families known today. In addition, these viruses are found in invertebrates, algae, fungi, and bacteria (Levy *et al.* 1994; Matthews 1991). Their genomes, consisting of one or more single stranded RNAs, are positive sense, meaning that they act as messenger RNAs (mRNAs) for the

translation of viral proteins after the virus successfully infects a host cell. The RNA genomes are surrounded by a protein coat called the capsid, which is specific for that virus and, especially in the animal viruses, the capsid may be surrounded by a lipid-rich envelope.

Many of these viruses are of medical concern and a large number of them have an agricultural impact as well. Table 2 lists some of the positive sense single stranded viruses known to infect vertebrates, plants, and bacteria, along with their taxonomic classification. Many of the plant viruses are not currently classified by family, but instead, are given a group classification.

Table 2
Examples of some single stranded positive sense RNA viruses.

Family Name	Genus name	Examples
		PROKARYOTIC
Leviviridae	*Levivirus*	MS2; R17; f2
	Allolevivirus	Qβ
		EUKARYOTIC -- Animal
Picornaviridae	*Enterovirus*	Polioviruses
	Rhinovirus	Rhinoviruses
	Aphthovirus	Foot-and-mouth disease virus
	Cardiovirus	Encephalomyocarditis virus
	Hepatitis A	Hepatitis A virus
	Unassigned	Equine rhinovirus 1 and 2; *Drosophila* P virus
Togaviridae	*Alphavirus*	Sindbis; Semliki forest virus; eastern equine encephalitis virus
	Rubivirus	Rubella virus (German measles)
	Arterivirus	Simian hemorrhagic fever virus
Flaviviridae	*Flavivirus*	Yellow fever virus; dengue; Japanese encephalitis virus
	Pestivirus	Bovine diarrhea virus; hog cholera virus
	Hepatitis virus C	Hepatitis virus C
Coronaviridae	*Coronavirus*	Avian infectious bronchitis virus; feline infectious peritonitis
Toroviridae	*Torovirus*	Berne virus
Caliciviridae	*Calicivirus*	Norwalk viruses; hepatitis E virus
Nodaviridae	*Nodavirus*	Black beetle virus; flock house virus
Tetraviridae	*Tetravirus*	
Unclassified	*Astrovirus*	

Table 2 -Continued

EUKARYOTIC -- Plant	
Alfamovirus	Alfalfa mosaic virus
Bromovirus	Brome mosaic virus
Capillovirus	Apple stem grooving virus
Carlavirus	Potato virus M
Carmovirus	Turnip crinkle virus
Closterovirus	Citrus tristeza virus
Comovirus	Cowpea mosaic virus
Cucumovirus	Cucumber mosaic virus
Dianthovirus	Red clover necrotic mosaic virus
Fabavirus	Broad bean wilt virus
Furovirus	Soil-borne wheat mosaic
Hordeivirus	Barley stripe mosaic
Ilarvirus	Tobacco streak virus
Luteovirus	Potato leafroll virus
Machlovirus	Maize chlorotic dwarf
Marafivirus	Maize rayado fino
Necrovirus	Tobacco necrosis virus
Nepovirus	Tomato black ring virus
Parsnip yellow fleck	Parsnip yellow fleck
Pea enation mosaic	Pea enation mosaic virus
Potexvirus	Potato virus X
Potyvirus	Tobacco etch virus
Sobemovirus	Southern bean mosaic virus
Tobamovirus	Tobacco mosaic virus
Tobravirus	Tobacco rattle virus
Tombusvirus	Tomato bushy stunt virus
Tymovirus	Turnip yellow mosaic virus

These viruses have a variety of replication and translation strategies (Levy *et al.* 1994; Matthews 1991), but, in general, they undergo the following infection process. The virion (a single, intact virus particle) attaches to the outside of the host cell and the viral genomic RNA enters the cell either as naked RNA or as RNA complexed with other viral components. Sometimes, as is seen with tobacco mosaic virus (TMV), the entire virion enters the cell. Once inside the host cell, viral proteins are translated and the genomic RNA is replicated. One of the first viral proteins translated is generally the viral replicase, which is then responsible for replication of the viral genome. Eventually progeny virions are assembled within the host cell and released from the cell to begin another infection cycle.

FIG. 1 details the general scheme of positive sense single stranded RNA virus replication. During replication, the viral replicase attaches to the 3' end of the positive sense genomic RNA and begins transcribing a negative sense RNA strand. It is possible that several replicases will be able to follow one another on the positive sense template strand. The negative sense strands, in turn, act as templates for synthesis of new positive sense strands. The new positive sense strands either act as new templates for additional negative strand synthesis or are translated into one or more viral proteins. Each virus species has its own particular translation strategy.

It has been observed that two different forms of viral RNA can be isolated from infected cells (Levy *et al.* 1994; Matthews 1991): the replicative form (RF) and the replicative intermediate form (RI). Whether these two forms are indeed occurring in an intact infected cell or whether they are simply artifacts of the extraction process is unknown (Levy *et al.* 1994). The RF RNA is double stranded and may represent a newly synthesized negative strand completely complexed with its positive sense template (see FIG. 1B). The RI form, on the other hand, is partially single stranded and partially double stranded. It may represent the situation depicted in FIG. 1A or 1C.

Several important replication and translation regulatory sequences are found in the 5' untranslated region (the omega sequence) of TMV. In addition, just downstream of the omega sequence is the translation start codon for the viral replicase. Therefore, the 5' end of TMV's genomic RNA is an attractive target for antisense design.

In the present invention, two antisense sequences complementary to nucleotides (nt) 1-192 and nt 379-581, respectively, of the positive sense TMV genomic RNA (SEQ ID NO:1) were fused so that they would theoretically cause the formation of a loop when the full length antisense RNA hybridized to TMV positive sense genomic RNA within an infected plant cell (FIG. 3). It is expected that this loop would prevent ribosomal recognition of the viral cap and/or the translation regulatory sequences within the omega sequence so that translation of the viral replicase would be inhibited. The loop structure might also possibly prevent

transcription of the full-length negative sense intermediate necessary for successful viral replication. Inhibition of translation or replication may also occur via an endonuclease-sponsored degradation of the RNA, with the formation of the loop being a better inducer of endonuclease attack that normal antisense binding.

The 3' end of the negative sense TMV strand is the exact complement of the 5' end of the positive sense strand. Thus a second set of two antisense sequences, complementary to the first antisense pair and thus complementary to the 3' end of the negative sense TMV RNA strand were fused to form a 395-nucleotide antisense molecule theoretically capable of causing loop formation at the 3' end of the negative sense TMV RNA. The negative sense strand serves as a template for replication of new genomic RNAs and all subgenomic RNAs produced by TMV, so this second antisense molecule might be able to inhibit TMV replication.

III. Target Genes

Gene therapy has become an increasingly viable endeavor in the past decade for the mere reason that genetic defects responsible for numerous genetic diseases have been identified. Such genes include cytokines, hormones, transporters, enzymes and receptors. Examples include the genes responsible for cystic fibrosis (CF), surfactant protein B deficiency and alpha-1-antitrypsin deficiency. Additionally, various antisense oncogene constructs, tumor suppressor genes, inducers of apoptosis, repair genes and toxins have been identified as potential therapeutics in various cancers. Particular oncogenes that are targets for antisense constructs are *ras, myc, neu, raf, erb, src, fms, jun, trk, ret, hst, gsp, bcl-2* and *abl*. Targets for this embodiment will include angiogenic genes such as VEGFs and angiopoeiteins as well as the oncogenes (*e.g., ras, myc, neu, raf, erb, src, fms, jun, trk, ret, hst, gsp, bcl-2, EGFR, grb2* and *abl*. A list of other potential therapeutic genes is set forth below.

A. Tumor Suppressors

Tumor suppressors that may be employed according to the present invention include p21, p15, BRCA1, BRCA2, IRF-1, PTEN, RB, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, FCC, p53, CDK's and MCC.

B. Inducers of Apoptosis

Inducers of apoptosis, such as Bax, Bak, Bcl-X_s, Bad, Bim, Bik, Bid, Harakiri, Ad E1B, Bad and ICE-CED3 proteases, similarly could find use according to the present invention.

C. Enzymes

Various enzyme genes are of interest according to the present invention. Such enzymes include for example, human copper zinc superoxide dismutase (U.S. Patent No. 5,196,335), cytosine deaminase, adenosine deaminase, hypoxanthine-guanine phosphoribosyltransferase, galactose-1-phosphate uridyltransferase, phenylalanine hydroxylase, glucocerbrosidase, sphingomyelinase, α-L-iduronidase, glucose-6-phosphate dehydrogenase, β-glucuronidase, HSV thymidine kinase and human thymidine kinase and extracellular proteins such as collagenase and matrix metalloprotease.

D. Transcription Factors and Regulators

Another class of genes that can be applied in an advantageous combination are transcription factors, for example C/EBPα, IκB, NfκB and Par-4.

E. Cell Cycle Regulators

Cell cycle regulators provide possible advantages, when combined with other genes. Such cell cycle regulators include for example, p27, p16, p21, p57, p18, p73, p19, p15, E2F-1, E2F-2, E2F-3, p107, p130 and E2F-4. Other cell cycle regulators include anti-angiogenic proteins, such as soluble Flt1 (dominant negative soluble VEGF receptor), soluble Wnt receptors, soluble Tie2/Tek receptor, soluble hemopexin domain of matrix metalloprotease 2 and soluble receptors of other angiogenic cytokines (*e.g.*, VEGFR1/KDR, VEGFR3/Flt4, both VEGF receptors).

IV. Administration and Pharmaceutically Acceptable Carrier

In clinical applications, it will be necessary to prepare the antisense compositions of the present invention as pharmaceutical compositions, *i.e.* in a form appropriate for *in vivo* applications. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

The compositions may be administered *via* any suitable route, including parenterally or by injection. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial an antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the antisense constructs in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of

sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration.

In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, a unit dose could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of

hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

V. Plant Transformation

In certain embodiments, the invention concerns transgenic plants and methods for the creation thereof. Suitable methods for plant transformation for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), by electroporation (U.S. Patent No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaeppler *et al.*, 1990; U.S. Patent No. 5,302,523, specifically incorporated herein by reference in its entirety; and U.S. Patent No. 5,464,765, specifically incorporated herein by reference in its entirety), by *Agrobacterium*-mediated transformation (U.S. Patent No. 5,591,616 and U.S. Patent No. 5,563,055; both specifically incorporated herein by reference) and by acceleration of DNA coated particles (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,877; and U.S. Patent No. 5,538,880; each specifically incorporated herein by reference in its entirety), *etc.* Through the application of techniques such as these, cells of virtually any plant species may be stably transformed, and these cells developed into transgenic plants.

A. Electroporation

Where one wishes to introduce DNA by means of electroporation, it is contemplated that the method of Krzyzek *et al.* (U.S. Patent No. 5,384,253, incorporated herein by reference in its entirety) will be particularly advantageous. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes,

are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells are made more susceptible to transformation by mechanical wounding.

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.*, 1995; D'Halluin *et al.*, 1992), wheat (Zhou *et al.*, 1993), tomato (Hou and Lin, 1996), soybean (Christou *et al.*, 1987) and tobacco (Lee *et al.*, 1989).

One also may employ protoplasts for electroporation transformation of plants (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in Intl. Patent Appl. Publ. No. WO 9217598 (specifically incorporated herein by reference). Other examples of species for which protoplast transformation has been described include barley (Lazerri, 1995), sorghum (Battraw *et al.*, 1991), maize (Bhattacharjee *et al.*, 1997), wheat (He *et al.*, 1994) and tomato (Tsukada, 1989).

B. Microprojectile Bombardment

A suitable method for delivering transforming DNA segments to plant cells in accordance with the invention is microprojectile bombardment (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610,042; and PCT Application WO 94/09699; each of which is specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold.

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with monocot plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Application WO 95/06128), barley (Ritala *et al.*, 1994; Hensgens *et al.*, 1993), wheat (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety), rice (Hensgens *et al.*, 1993), oat (Torbet *et al.*, 1995; Torbet *et al.*, 1998), rye (Hensgens *et al.*, 1993), sugarcane (Bower *et al.*, 1992), and sorghum (Casas *et al.*, 1993; Hagio *et al.*, 1991); as well as a number of dicots including tobacco (Tomes *et al.*, 1990; Buising and Benbow, 1994), soybean (U.S. Patent No. 5,322,783, specifically incorporated herein by reference in its entirety), sunflower (Knittel *et al.* 1994), peanut (Singsit *et al.*, 1997), cotton (McCabe and Martinell, 1993), tomato (VanEck *et al.* 1995), and legumes in general (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety).

C. *Agrobacterium*-mediated Transformation

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant

cells is well known in the art. See, for example, the methods described by Fraley *et al.*, (1985), Rogers *et al.*, (1987) and U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety.

Agrobacterium-mediated transformation is most efficient in dicotyledonous plants and is the preferable method for transformation of dicots, including *Arabidopsis*, tobacco, tomato, and potato. Indeed, while *Agrobacterium*-mediated transformation has been routinely used with dicotyledonous plants for a number of years, it has only recently become applicable to monocotyledonous plants. Advances in *Agrobacterium*-mediated transformation techniques have now made the technique applicable to nearly all monocotyledonous plants. For example, *Agrobacterium*-mediated transformation techniques have now been applied to rice (Hiei *et al.*, 1997; Zhang *et al.*, 1997; U.S. Patent No. 5,591,616, specifically incorporated herein by reference in its entirety), wheat (McCormac *et al.*, 1998), barley (Tingay *et al.*, 1997; McCormac *et al.*, 1998), and maize (Ishidia *et al.*, 1996).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate the construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987) have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

D. Other Transformation Methods

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, *e.g.*, Potrykus *et al.*, 1985; Lorz *et al.*, 1985;

Omirulleh *et al.*, 1993; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts have been described (Fujimara *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986; Omirulleh *et al.*, 1993 and U.S. Patent No. 5,508,184; each specifically incorporated herein by reference in its entirety). Examples of the use of direct uptake transformation of cereal protoplasts include transformation of rice (Ghosh-Biswas *et al.*, 1994), sorghum (Battraw and Hall, 1991), barley (Lazerri, 1995), oat (Zheng and Edwards, 1990) and maize (Omirulleh *et al.*, 1993).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989). Also, silicon carbide fiber-mediated transformation may be used with or without protoplasting (Kaeppler, 1990; Kaeppler *et al.*, 1992; U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety). Transformation with this technique is accomplished by agitating silicon carbide fibers together with cells in a DNA solution. DNA passively enters as the cell are punctured. This technique has been used successfully with, for example, the monocot cereals maize (PCT Application WO 95/06128, specifically incorporated herein by reference in its entirety; Thompson, 1995) and rice (Nagatani, 1997).

VI. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be

made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1
Materials and Methods

Plants and Viruses

Transgenic *Nicotiana tabacum* cv. Xanthi NN and Xanthi nn were the local lesion and systemic tobacco plant hosts, respectively, for TMV-U1. The local lesion response is a natural resistance response which allows quantification of viral replicative success in the presence or absence of transgenes by measuring the size and numbers of lesions. The systemic response represents natural nonresistance. Inhibition of viral replication in transgenic systemic plants is measured by delay in onset of symptoms. TMV strain U1 was propagated in nontransgenic *N. tabacum* cv. Xanthi nn. Virions were extracted and purified according to the protocol of Gooding and Hebert (1967).

Plasmid Construction

Nucleotide position designations represent positions within TMV, numbered according to Goelet (1982). The cloning procedures used are as previously described (Ausubel, 1998) with minor modifications. All PCR primers used in transgene construction are characterized in Table 3. Plasmids were propagated in *Escherichia coli* strain DH5α (Life Technologies, Inc.; Grand Island, NY) except for pRK2013, which was propagated in *E. coli* strain MM294. Maxi- and minipreps of each plasmid were done according to the alkaline lysis method of Birnboim (1983) using a polyethylene glycol precipitation (Lis, 1980) or hand-packed Sepharose® (Pharmacia Biotech, Inc.; Piscataway, NJ) columns. The orientation and integrity of cloned inserts were checked by appropriate restriction digests and/or PCR analyses. Clones were prepared for PCR screening using a boiling lysis method. Briefly, a small amount of each colony was dispersed in 50 μl of water and placed in a boiling water bath for 5 minutes. Each sample (1 μl) was then added to a PCR mix. PCR products and restriction digests were electrophoresed on agarose gels and the DNA was purified from excised gel bands using sodium iodide and silica powder (GeneClean™; BIO 101, Inc.; La Jolla, CA).

Table 3

Primer Code	Sequence (5' to 3')	SEQ ID NO	Restriction Sites
T83	CGG.TCA.CGA.GCT.CTA.AAC.TCT.TC	2	*Sac*I
T84	CCC.TAC.GGT.ACC.TTG.ACT.TAT.GAC	3	*Kpn*I
T85	ATA.TGA.ATT.CGT.ATT.TTT.ACA.ACA.ATT.ACC .AAC.AAC	4	*Eco*RI
T86	GTC.TTC.AGG.AAT.TCG.TGC.GTA.TCT.GT	5	*Eco*RI
AK36	CAC.TAT.CTC.GAG.GAT.CCG.GTA.CCT.TGA.CTT .ATG.ACA	6	*Xho*I, *Bam*HI, *Kpn*I
A46	GGA.TCC.CTC.GAG.TCT.AGA.GAA.TTC.GTG.CG T.ATC.TGT	7	*Xho*I, *Xba*I, *Eco*RI
AS36	AAG.GGA.CTC.GAG.TCT.AGA.GCT.CTA.AAC.TC T.TCA.ACC	8	*Xho*I, *Xba*I, *Sac*I
A35	TCG.ATA.CTC.GAG.GAT.CCG.AAT.TCG.TAT.TTT .TAC.AAC	9	*Xho*I, *Bam*HI, *Eco*RI

pT7TMV, containing the cDNA copy of the TMV-U1 genomic RNA, was amplified with primers T85 and T83 to produce a 209 bp product (AL83/85) representing nt 1-192 of TMV-U1. A different amplification using primers T84 and T86 produced a 231 bp product (AL84/86) representing nt 379-581 of TMV-U1. Purified DNA was then digested with either *Sac*I and *Eco*RI (AL83/85) or *Kpn*I and *Eco*RI (AL84/86). Digestion products were ligated into pBluescript® SK- (pBSK) (Stratagene; La Jolla, CA) and cloned to produce pBSK-AL83/85 and pBSK-AL84/86, respectively. The full-length fragment (AL83/86) was constructed by digesting the previously cloned plasmids with *Sac*I and *Eco*RI (pBSK-83/85) or *Kpn*I and *Eco*RI (pBSK-84/86). The restriction fragments were ligated together, and then ligated into pBSK which had been digested with *Kpn*I and *Sac*I.

pBSK-AL clones were amplified with primers carrying appropriate restriction enzyme sites (see Table 3) to allow insertion of AL fragments into a pRTL2 vector (Topfer *et al.* 1987) carrying the β-glucuronidase gene (GUS). pRTL2-GUS was kindly provided by Pal Maliga. The GUS marker gene was removed during the digests done to prepare pRTL2-GUS for AL insertion. AL PCR products were digested, and then ligated into pRTL2, followed by cloning, to produce pRTL-AL83/85 (+), pRTL-AL83/85 (-), pRTL-AL84/86 (+), pRTL-AL84/86 (-), pRTL-AL83/86 (+), and pRTL-AL83/86 (-) (Table 4).

Table 4

Construction of pRTL2-AL Clones			Construction of pPZP-AL Clones		
PCR Template	Primers[a]	Digest[b]	pRTL Clone	Fragment Size[c]	pPZPClone
pBSK-AL83/86+	AK36, AS36 (442)	*Xho*I, *Xba*I (422)	pRTL2-AL83/86+	1393	pPZP-AL83/86 +
pBSK-AL83/86 -	AK36, AS36 (442)	*Xho*I, *Bam*HI (423)	pRTL2-AL83/86 -	1400	pPZP-AL83/86 -
pBSK-AL83/85+	A35, AS36 (231)	*Xba*I, *Eco*RI (200)	pRTL2-AL83/85+	1176	pPZP-AL83/85 +
pBSK-AL83/85 -	A35, AS36 (231)	*Xho*I, *Bam*HI (212)	pRTL2-AL83/85 -	1189	pPZP-AL83/85 -
pBSK-AL84/86+	AK36, A46 (252)	*Xho*I, *Xba*I (232)	pRTL2-AL84/86+		
pBSK-AL84/86 -	AK36, A46 (252)	*Xho*I, *Xba*I (221)	pRTL2-AL84/86 -		

[a]Numbers in ( ) represent sizes of PCR products obtained with the indicated primers.

[b]PCR products were digested with the indicated restriction enzymes. Restriction fragments of the size indicated in ( ) were then ligated into the pRTL2 plasmid vector, giving the pRTL2-AL clones listed in column 4.

[c]pRTL2-AL clones were restricted with *Pst*I to give the indicated fragment sizes in bp. These fragments were then ligated into a pPZP plasmid vector.

The pRTL2-AL clones, except for pRTL2-AL84/86 (+) and pRTL2-AL84/86 (-), were digested with *Pst*I and ligated into pPZP111 (Hajdukiewicz *et al.* 1994) (kindly provided by Pal Maliga) to produce pPZP-AL83/86 (+), pPZP-AL83/86 (-), pPZP-AL83/85 (+), and pPZP-AL83/85 (-) (see Table 4). pRTL2-AL84/86 (+) was digested with *Hin*dIII and the 4283 bp fragment containing the TMV sequence was ligated into pPZP-AL83/85 (+), producing pPZP-AL83/85-84/86 (+). Similarly, pPZP-AL83/85-84/86 (-) was constructed by digesting pRTL2-AL84/86 (-) with *Hin*dIII and ligating it into pPZP-AL83/85 (-). All pPZP-AL clones were grown on LB plating media containing 25 μg chloramphenicol per ml.

The helper plasmid, pRK2013 (Bevan 1984) in *E. coli* strain MM294, was propagated on LB medium containing 50 μg of kanamycin sulfate per ml. *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.,* 1983), was grown on minimal A medium containing 100 μg of streptomycin per ml.

Triparental Mating and Plant Transformation

Triparental matings (Rogers *et al.* 1986) and plant transformations were performed according to the protocol of Svab *et al.* (1995). Triparental matings utilized *A. tumefaciens* strain LBA4404 (Ooms *et al.* 1982), *E. coli* strain MM294 (pRK2013), and each of the *E. coli* strain DH5α (pPZP-AL) clones individually. pPZP111 containing no TMV sequence was also mobilized into *A. tumefaciens* to act as a control. Successfully transformed *A. tumefaciens* clones were identified by growth on minimal A medium containing 25 μg of chloramphenicol per ml and then were single-cell purified.

Leaf pieces (1 cm^2) from *Nicotiana tabacum* cv. Xanthi NN and Xanthi nn were individually cocultivated with *A. tumefaciens* strain LBA4404 (pPZP-AL) or (pPZP111) clones on Murashigi and Skoog (MS) medium containing 100 μg kanamycin sulfate per ml. Shoots were excised and transferred to MS medium containing kanamycin sulfate (100 μg per ml), 1-naphthaleneacetic acid (NAA) (0.1

μg per ml), and 6-benzylaminopurine (BAP) (1 μg per ml) to promote root formation. Shoots that rooted successfully were potted in soil and transferred to the greenhouse.

Extraction of Transgene RNA

Tissue from unexpanded leaves taken from each unique transgenic *N. tabacum* cv. Xanthi nn line was crushed in a liquid nitrogen-cooled microfuge tube using sterile drill-driven plastic pestles. Samples were frozen at -80°C until the addition of the extraction solution. Total RNA was extracted using TriReagent® (Molecular Research Center, Inc.; Cincinnati, OH) according to the manufacturer's instructions (Chomczynski 1993). RNA pellets were resuspended in 25 μl of 0.1 mM sodium citrate and treated with RNase-free DNase (2 units) for 30 minutes at 37°C. Each sample was heated to 65°C for 5 minutes to inactivate the DNase and was then frozen at -80°C.

RNA Normalization

In order to standardize the relative amounts of total RNA in each transgenic plant sample, 28S rRNA band intensities on ethidium bromide-stained gels were measured under UV light. Each RNA sample (8 μl) was mixed with 2 μl of loading buffer (10 mM sodium phosphate, pH 7.0; 0.25% bromphenol blue; 50% glycerol) and 1 μl of 500 ng/ml ethidium bromide, and then incubated at 65°C for 15 minutes. Samples were then chilled on ice for two minutes and loaded onto a 1.2% agarose gel prepared with 10 mM sodium phosphate buffer, pH 7. RNase-free conditions were maintained throughout the procedure. The gel was run in 1X MOPS buffer (0.2 M 3-(N-morpholino)-propanesulfonic acid, pH 7; 0.5 M sodium acetate; 0.01 M EDTA). Samples were normalized by comparing 28S rRNA band intensities under UV light (Berde *et al.* 1992; Bonini and Hofmann 1991) using the public domain NIH Image program (written by Wayne Rasband at the U. S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868).

RNA Slot Blots and Hybridization

Slot blots were run with normalized samples and appropriate controls essentially according to the manufacturer's directions (Bio-Rad Laboratories; Hercules, CA). Probe preparation and blot hybridization were done according to the Genius™ System protocols (Boehringer-Mannheim Corp.; Indianapolis, IN). Blots were hybridized with digoxygenin-labeled probes diluted in high SDS prehybridization solution (25 ng probe per ml) at 50°C overnight. Primers A35 and AS36 (pPZPAL-83/85 (-) template) were used to make one probe (A3/5) and primers AK36 and A46 (pPZPAL-84/86 (-) template) were used to make the other probe (A4/6). Densitometry analyses of blots were done using the NIH Image program.

Plant Cloning

Leaf pieces from each unique transgenic plant line were tissue cultured on MS medium containing kanamycin sulfate (100 μg/ml) using sterile techniques described in the protocol of Svab *et al.* (1995). Shoots were excised and transferred to MS medium containing kanamycin sulfate (100 μg/ml), NAA (0.1 μg/ml), and BAP (1 μg/ml). Rooted shoots were potted in soil and kept under fluorescent lights at 21°C.

Inoculation of Plants

In the screening trials, 200 ng of TMV virions/μl of FES buffer (100 mM glycine pH 9.2, 60 mM K_2HPO_4, 1% sodium pyrophosphate, 1% diatomaceous earth, 1% Bentone MA (RHEOX, Inc.; Hightstown, NJ)) was manually inoculated onto each of two leaves per plant by gentle rubbing. Transgenic *N. tabacum* cv. Xanthi nn plants were monitored for 30 days in the greenhouse for the first appearance of systemic symptoms in the newest upper leaves: vein clearing and/or a mosaic pattern. The plant lines that showed resistance were tissue cultured again to obtain additional test clones. These clones (approximately 6 to 10 cm tall) were challenged with TMV (42 μg of virions/plant) and monitored for systemic symptom development for 30 days. Leaf samples from a few of the plants that were asymptomatic at the end of 30 days were crushed in FES buffer to make a slurry, which was then inoculated onto nontransgenic *N. tabacum* cv. Xanthi NN plants. Infection was indicated by the appearance of lesions.

The first four fully-expanded leaves on each of three to six *N. tabacum* cv. Xanthi NN clones from each line (approximately 11 cm tall) were manually inoculated with 50 μl of TMV per half leaf (TMV was diluted in FES buffer to give approximately 50 lesions per inoculation on nontransgenic *N. tabacum* cv. Xanthi NN plants). Lesions on all four leaves (eight half leaves) were counted at 60 hours post inoculation and randomly chosen lesions on leaves two and four were measured at 90 hours post inoculation. The time of first appearance of lesions was also noted for each of the eight half leaves on each plant.

Example 2
Construction of Antisense Plasmids for Plant Transformation

Ten sets of plants were independently transformed using Agrobacterium-mediated transformation with pPZP-TMV-U1 sequences or with pPZP vector sequences only were produced using either *Nicotiana tabacum* cv. Xanthi nn (systemic host; five sets) or Xanthi NN (local lesion host; five sets) plant tissue. Each set of plants was then challenged with TMV-U1 and monitored for inhibition of TMV multiplication. The full-length transgene, designated AL83/86, carried nt 1-192 and nt 379-581 of the TMV-U1 genome. The first antisense domain (nts. 1-192) corresponds to the entire 5' nontranslated region of tmv, the initiation aug (nt. 69) and a portion of the replicase coding region. The second antisense domain corresponds to a portion of the tmv replicase coding region. These two TMV sequences were fused (see FIG. 4A and FIG. 4B) and then were ligated into the pPZP111 *Agrobacterium* binary vector in a sense (+) orientation to produce pPZP-AL83/86 (+) (FIG. 4A). This same full-length transgene was also ligated into pPZP111 in an antisense (-) orientation to produce pPZP-AL83/86 (-). Both fusion transgenes were under the control of a dual 35S promoter from cauliflower mosaic virus (CaMV) and had a CaMV polyadenylation signal downstream.

To determine whether the separate arms of the fusion transgene could inhibit TMV multiplication equally as well as the putative loop-inducing fusion transgene, two other pPZP-AL plasmids were constructed and used in a triparental mating and plant transformation. Each of the arms of a full-length fusion transgene was

independently ligated into a single pPZP111 vector to produce pPZP-AL83/85-AL84/86 (in either sense or antisense orientation), each arm having its own dual 35S promoter and polyadenylation signal (see FIG. 4B). The full-length and separate arm transgenes (in both sense and antisense orientation), as well as a pPZP111 vector containing no TMV sequences, were then mobilized into *Agrobacterium tumefaciens* strain LBA4404, producing 4 different *A. tumefaciens* cultures which were subsequently used to transform both *Nicotiana tabacum* cv. Xanthi nn and Xanthi NN. Table 5 lists the ten plant categories mentioned above and the numbers of unique plant lines obtained within each category. . A "unique plant line" was considered as derived from a single shoot as the sole representative from a well separated area of plant tissue transformed with *A. tumefaciens* (pPZP-AL) or *A. tumefaciens* (pPZP) early in the culturing process. Only shoots able to root on kanamycin sulfate-containing media were used.

Table 5. Transgenic plant categories

pPZP Vector[a]	*N. tabacum* cv. Xanthi nn[d]		*N. tabacum* cv. Xanthi NN[e]	
	Plant Category[b]	Number of Lines[c]	Plant Category[b]	Number of Lines[c]
pPZP-AL83/86 (+)	TFP	38	GFP	28
pPZP-AL83/86 (-)	TFN	20	GFN	13
pPZP-AL83/85-AL84/86 (+)	TQP	22	GQP	17
pPZP-AL83/85-AL84/86 (-)	TQN	23	GQN	18
pPZP	Tzero	16	Gzero	12

[a]pPZP vector with or without TMV sequences which was mobilized into *A. tumefaciens* strain LBA4404 and was then used to transform appropriate plant tissue.

[b]T = *N. tabacum* cv. Xanthi nn; G = *N. tabacum* cv. Xanthi NN; F = full-length fusion transgene; Q = two arms of full-length transgene under separate CaMV promoters; P = positive sense; N = negative sense.

[c]Each plant line represents a unique transformation event.

[d]Source of plant tissue used in plant transformation procedures.

Transgene RNA Levels Determined

In order to determine relative transgene expression levels for each plant line, samples of total RNA from each line were blotted and hybridized with a probe that recognized the transgene sequence. Since the total RNA content of each sample could vary greatly depending upon RNA extraction efficiency and the starting amount of plant material, an RNA normalization gel was run prior to slot blotting and hybridization. Ethidium bromide-stained, agarose gel loaded with 8 µl of each RNA sample from the TFP plant lines was used to normalize total RNA. The 28S rRNA band densities were compared to determine the relative amount of 28S rRNA (and thus, total RNA) in each sample. Based on these determinations, equivalent aliquots of total RNA were then blotted and hybridized. Band intensities were analyzed by densitometry using a TMV RNA dilution series as the standard from which transgene RNA levels were estimated. FIG. 5 and FIG. 6 show the TFP and TFN transgene expression levels for each unique plant line in the two categories. For both the TFP and the TFN plants, most transgene RNA expression levels appear to be below 100 pg of transgene RNA per 7.5 µg of total RNA.

Example 3
TMV Resistance of Each Systemic Plant Line

Because of the large number of plant lines obtained for each system host category (TFP, TFN, TQP, TQN, Tzero), an initial screening was done to determine which plant lines in each category were most resistant to TMV infection. Many of these plant lines had delayed or no seed production, so it was decided that each line would be cloned by culturing leaf pieces on shoot-inducing media. The resulting shoots were then rooted and transferred to soil. Each shoot in a particular plant line was presumed to be genetically identical. Although it was realized that somaclonal

variation might arise in a certain number of plants, the risk of this happening was considered acceptably low. The number of clones produced for each plant line and screened by TMV inoculation generally ranged from 10 to 15 plants. Each plant was inoculated with TMV and monitored for 30 days to detect symptoms of systemic infection. At least five plant lines that exhibited the most delay in symptom development within each category were chosen for a larger scale repeat of the screening trials. Some categories such as TQP and Tzero had not gone through the initial TMV screening process, so several more lines from these categories were included in the large-scale TMV challenge trials. Table 6 lists the plant lines and the number of clones in each line that were rechallenged with TMV.

Table 6. Number of clones per plant line per category used in the large scale TMV challenge trials.

Category	TFP								TFN						
Plant line	21	26	28	29	30	31	32	33	1	8	9	10	11	12	14
No. of clones	25	24	20	10	28	29	39	35	36	28	10	22	18	31	21
Category	TQP														
Plant line	5	6	7	9	10	11	13	14	16	20	24	25	26		
No. of clones	15	27	15	22	19	15	26	26	10	20	23	34	21		
Category	TFN					Tzero									
Plant line	2	4	8	9	10	3	4	7	8	9	10	11	12	18	19	21
No. of clones	15	20	32	8	22	25	17	15	7	27	20	19	30	28	21	26

Since there is a high degree of line-to-line variability in the expression of plant transgenes, a large number of plant lines representing each transgene had to be examined to compare the most TMV-resistant lines of each transgenic category. FIG. 7, FIG. 8, FIG. 9, FIG. 10, and FIG. 11 show the percentage of plants in each plant line of each category that exhibited symptoms of systemic infection at various days post inoculation. From the this data, it can be seen that tfn lines 8, 9, and 12, showed greater delay in symptom development over the 30 day monitoring period

(FIG. 8) than any other plant line from the tq or tzero controls (FIGS. 9-11). These lines had a lesser proportion of plants infected at either the middle or the end of the 30-day monitoring period. Symptom delay also occurred with the tfp 21 line in the middle of the monitoring period (FIG 7). Two of the T_0 plant lines (T_0 18 and 21) had a slight delay in symptom development as compared with the TQP and TQN plant lines, but this delay was not as great as that seen in TFP21, TFN8, and TFN9, for example. During these trials, greenhouse temperatures ranged from 20-33°C, with an average daylight temperature of around 30°C.

In the initial 30 day screening trials, none of the nine TFN9 clones developed symptoms. These nine plants were then reinoculated with TMV twice more over the next 60 days and did not develop symptoms of TMV infection during that time. These screening trials were conduced in the spring, with greenhouse temperatures ranging from 13-26°C, with an average daylight temperature of 23°C. The first appearance of symptoms during the initial screening trials was around day 10 post inoculation, but first appearance of symptoms was around day 6 post inoculation during the final trials, which were held in the summer, when average greenhouse temperatures reached 30°C. Thus the final trials were more stringent than the screening trials and may have reduced the differences between the treatments.

Twelve plants in the TFP and TFN categories were asymptomatic at 30 days post inoculation. To determine whether or not asymptomatic plants were carrying a "hidden" TMV infection, pieces of the apical leaves from six of these plants were collected and individually ground up in FES buffer to make slurries. These slurries were then inoculated onto a nontransgenic local lesion host (*N. tabacum* cv. Xanthi NN) and the resulting lesions were counted (Table 7). All six asymptomatic plants were infected with TMV. The TFN9 and 12 plants, however, had a very low TMV titer in the new apical leaves.

Table 7. Lesion counts from selected asymptomatic transgenic *Nicotiana tabacum* cv. Xanthi nn clones

Clone	TFP 30-6	TFN 9-5	TFN 9-10	TFN 11-23	TFN 12-14	TFN 12-18
Number of lesions[a]	>200	36	41	>200	11	21

[a]Leaf tissue from apical leaves was ground in FES buffer and inoculated onto *Nicotiana tabacum* cv. Xanthi NN plants. Lesions were counted 4 days later.

The above data support the conclusion that the TFN transgene is better able to inhibit TMV replication and symptom development than the TQN, TQP, and Tzero transgenes (FIGS. 8-11). Thus, antisense with the two domains contiguous to each other on the same antisense mRNA molecule (TFN) better inhibits TMV replication than the antisense with the two domains on separate molecules (TQN). Since TFN is expected to induce loop formation while TQN is not, and since both transgenes contain the identical sequences, this indicates that loop-induction is important for inhibition of TMV replication. TFP transgenic line 21 appeared to delay symptoms during the monitoring period, but TFP line 30 did not prevent the accumulation of symptoms (Table 7). TFN would be expected to inhibit translation predominantly (binds to 5' end of (+) TMV RNA), and hence TMV replication. TFP would be expected to inhibit viral replication directly (binds to 3' end of (-) RNA). Finally, the efficacy of the TFN lines was demonstrated by the inhibition of the final accumulation of TMV (Table 7) and by line TFN9, which was completely resistant to TMV challenge in the Spring screening trials and showed unusual symptom delay and resistance in the fall final trials.

Example 4
TMV Resistance of Each Nonsystemic Plant Line

Transgenic *N. tabacum* cv. Xanthi NN plants in all five categories (GFP, GFN, GQP, GQN, and Gzero) were inoculated with TMV to determine if the transgene would inhibit either TMV's ability to initiate an infection or the cell-to-cell movement of TMV. Two aspects of infection initiation were (1) the time of the first appearance

of lesions on the inoculated leaf and (2) the number of lesions that actually develop. The first four fully expanded leaves of each test plant were inoculated with TMV. Each leaf was numbered according to its position on the plant, with leaf 1 being the top-most leaf. Leaf position determines the number of local lesions that will develop for a particular TMV inoculum concentration and the sizes of those lesions (Matthews 1991). Therefore, the data for each leaf position were not comparable to data associated with leaves at other positions on the plant.

FIG. 12 shows the average time of the first appearance of lesions on each leaf for each plant category. The Gzero plants showed a possible delay in local lesion appearance for every leaf position. All other plant categories showed no significant differences in times of first appearance of lesions.

The average number of lesions that developed at each leaf position for each plant category is represented in FIG. 13. There were no significant differences found among the four categories.

The relative rate of cell-to-cell movement was indicated by lesion diameters at 90 hours post inoculation. FIG. 14 shows the average lesion diameters for leaves 2 and 4 of ten GFP plant lines. Each bar represents the average lesion diameters (15 lesions per plant) for each of one to six plants inoculated in that particular line. Therefore, the data collection for the lesion diameters was discontinued.

FIG. 15 shows the average lesion diameters in each category (except for Gzero) for leaves 2 and 4. Again, there were possible differences between the GF and GQ categories but these differences were not significant and lesion diameter data collection was discontinued.

Example 5
Testing of Homozygous and Heterozygous R1 Plants Derived from R0 Plants

Transgenic tobacco plants containing test and control antisense sequences against tobacco mosaic virus (TMV) were screened for resistance to TMV. These

plants were the rooted shoots from transformed callus (R0 generation) and were therefore heterozygotes. These plants were self-pollinated to produce R1 generation plants. The R1 generation plants are self-pollinated to produce an R2 generation. The R1 generation is then screened for resistance to TMV using methods described above.

Prior to screening, a determination of which plants are heterozygous and which are homozygous for the transgenes is made by examining, by PCR using primers specific to the transgene, the proportions of R2 seedlings containing the transgene for each R1 specimen. An R2 ratio of roughly 3:1 (presence:absence of transgene) indicates that the R1 specimen from which the R2 is derived is a heterozygote, while an R2 population that was 100% positive for the transgene indicates that the parental R1 is homozygous for the transgene.

Heterozygotes were screened, but it is commonly found in such plant virus antisense studies that only the homozygotes show measurable levels of resistance. Though some resistant lines were found, especially with the TFN (potentially loop-inducing) antisense construct, increasing the resistance with all constructs will more clearly differentiate between different levels of resistance between different constructs. The initial screening was performed in the spring and yielded good resistance levels, but control plants were not included. The complete screening, with control plant lines, was conducted in the summer, resulting in the lower levels of resistance reported above.

Based on the above, screening with homozygote plants (and perhaps some heterozygote plants) during the spring months is expected to optimize resistance levels and better distinguish differential resistance induced by control and test antisense constructs.

Example 6
Testing Antisense Constructs in Plant Protoplasts Infected with TMV

The original antisense constructs were inserted in pBluescript cloning vectors to produce the pBSK-AL plasmid constructs. These constructs were then subcloned

to produce the pRTL2 and pPZP vectors for use in plant transformation. Table 4 of Example 1 above lists these plasmid constructs.

These original pBSK-AL plasmids will be used to produce RNA transcripts from the antisense sequence for testing inhibition of TMV replication in nontransgenic tobacco protoplasts. The transcripts will either be produced *in vitro* and coinoculated with the TMV RNA by electroporation of the protoplasts, and/or the transcripts and TMV RNA will be produced in the protoplasts by electroporation of the protoplasts to introduce transient expression vectors expressing said sequences. In addition, antisense oligonucletides, similar to those described in Example 10, may also be tested.

It is expected that the RNA transcripts from the antisense sequence will inhibit TMV replication in the tobacco protoplasts.

Example 7
Testing Antisense Constructs in Plant Protoplasts Infected with Other Viruses

The experiment described in Example 6 above may also be conducted with antisense sequences against other plant viruses, using protoplasts co-inoculated with RNA of other plant viruses via the methods outlined above. In addition, antisense oligonucletides, similar to those described in Example 10, may also be tested.

RNA transcripts from antisense sequences against other plant viruses will inhibit replication of other plant viruses in the tobacco protoplasts.

Example 8
Testing Plant Gene Antisense Constructs in Plant Protoplasts

Potentially loop-inducing antisense may also be tested against natural or transgenic genes harbored by plant protoplasts by electroporating either antisense RNA or antisense genes carried by transient expression vectors. Test and control constructs are similar in general design to those for TMV, as described above. In

addition, antisense oligonucletides, similar to those described in Example 10, may also be tested.

This study will show that these natural or transgenic genes in plant protoplasts are inhibited by the potentially loop-inducing antisense.

Example 9
Testing Antisense Constructs in Human or Animal Cell Culture

Experiments similar to those outlined in Examples 6, 7 and 8 may be carried out using human or animal cell cultures, according to methods known to those of skill in the art in view of the specification. In this case, human or animal cellular or viral RNA are the potential targets. In addition, antisense oligonucletides, similar to those described in Example 10, may also be tested in human or animal cell cultures. These studies will provide information on the manner in which antisense constructs affect human or animal cell cultures, and prove the therapeutic use of such constructs.

Example 10
Testing Antisense Oligonucleotides for Inhibition of *In Vitro* Translation

In order to test for efficacy of inhibition of *in vitro* translation by antisense sequences, antisense DNA oligonucleotides will be hybridized to test mRNA and the hybrid added to a rabbit reticulocyte or wheat germ *in vitro* translation assay. Potentially loop- inducing test sequences will be used as well as two antisense oligonucleotides binding to identical target sequences but not inducing a loop in the target mRNA, since they are unlinked (control antisense sequence). In this way, the test and control sequence design is analogous to the design in the examples above. The two antisense sequences will be 10-30 bases long, with a linker sequence in the oligonucleotide of 0-10 bases (or possibly composed of a non-nucleic acid polymer). The antisense oligonucleotides may consist of DNA or phosphorothioates or other modified nucleic acids. Antisense RNA, derived from *in vitro* transcription, may also be tested. In this case, each antisense RNA section will be 30-300 bases long. Target mRNAs may be viral or cellular, from plants, animals, humans or bacteria, with the antisense sequences being complementary to the target mRNAs.

* * * * * * * * * * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

PCT Application WO 9217598
PCT Application WO 94/09699
PCT Application WO 95/06128
PCT Application WO 95/06128
U.S. Patent No. 5,302,523
U.S. Patent No. 5,322,783
U.S. Patent No. 5,384,253
U.S. Patent No. 5,384,253
U.S. Patent No. 5,384,253
U.S. Patent No. 5,464,765
U.S. Patent No. 5,508,184
U.S. Patent No. 5,538,877
U.S. Patent No. 5,538,880
U.S. Patent No. 5,538,880
U.S. Patent No. 5,550,318
U.S. Patent No. 5,550,318
U.S. Patent No. 5,563,055
U.S. Patent No. 5,563,055
U.S. Patent No. 5,563,055
U.S. Patent No. 5,563,055
U.S. Patent No. 5,563,055
U.S. Patent No. 5,591,616
U.S. Patent No. 5,591,616
U.S. Patent No. 5,610,042

Abel, Nelson, De, Hoffmann, Rogers, Fraley, Beachy, "Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene," *Science,* 232(4751):738-743, 1986.

Arcone, *et al.*, *Nucl. Acids Res.*, 16(8):3195-3207, 1988.

Arya *et al.*, *Hum Gene Ther.* 9(9): 1371-1380, 1998

Ausubel, *Current Protocols in Molecular Biology*, New York: John Wiley and Sons, Inc., 1998.

Baichwal and Sugden, *In: Gene Transfer*, Kucherlapati R, ed., New York, Plenum Press, pp. 117-148, 1986.

Bartlett *et al.*, *Proc. Nat'l Acad. Sci. USA*, 93:8852-8857, 1996.

Bates, "Genetic transformation of plants by protoplast electroporation," *Mol Biotechnol.*, 2(2):135-145, 1994.

Battraw and Hall, "Stable transformation of sorghum-bicolor protoplasts with chimeric neomycin phosphotransferase II and beta glucuronidase genes," *Theor. App. Genet.*, 82(2):161-168, 1991.

Beachy, "Mechanisms and applications of pathogen-derived resistance in transgenic plants," *Curr. Opin. Biotechnol.*, 8:215-220, 1997.

Becker *et al.*, "Fertile transgenic wheat from microprojectile bombardment of scutellar tissue," *Plant J.*, 5(2):299-307, 1994.

Bennett, "Antisense oligonucleotides: is the glass half full or half empty?" *Biochem. Pharmacol.*, 55:9-19, 1998.

Benvenisty and Neshif, *Proc. Nat'l Acad. Sci. USA*, 83:9551-9555, 1986.

Berde, Griggs, Masters, "High sensitivity quantification of RNA from gels and autoradiograms with affordable optical scanning," *Biotechniques*, 12:902-911, 1992.

Bevan, "Binary Agrobacterium vectors for plant transformation, *Nucleic Acids Res.*, 12:8711-8721, 1984.

Bhattacharjee; An; Gupta, *J. Plant Bioch. and Biotech.* 6, (2):69-73. 1997.

Birnboim, "A rapid alkaline lysis extraction method for the isolation of plasmid DNA," *Methods Enzymol*, 100:243-255, 1983.

Blomer *et al.*, *J Virol.* 71(9): 6641-6649, 1997

Bonini and Hofmann, "A rapid, accurate, nonradioactive method for quantitating RNA on agarose gels," *Biotechniques*, 11(6):708-710, 1991.

Bower *et al.*, *The Plant Journal*, 2:409-416. 1992.

Brysch and Schlingensiepen, "Design and application of antisense oligonucleotides in cell culture, *in vivo*, and as therapeutic agents," *Cell Mol. Neurobiol.*, 14(5):557-68, 1994.

Buising and Benbow, "Molecular analysis of transgenic plants generated by microprojectile bombardment: effect of petunia transformation booster sequence," *Mol. Gen. Genet.*, 243:71-81, 1994.

Bull, Jacobson, Badgett, Molineux, "Viral escape from antisense RNA," *Mol. Microbiol.*, 28(4):835-46, 1998.

Callis *et al., Genes Dev.*, 1:1183-1200, 1987.

Carter and Flotte, *Curr. Top. Microbiol. Immunol.*, 218:119-144, 1996.

Casas *et al.*, "Transgenic sorghum plants *via* microprojectile bombardment," *Proc. Natl. Acad. Sci. USA*, 90(23):11212-11216, 1993.

Chang *et al.*, *Hepatology*, 14:124A, 1991.

Chatterjee, *et al., Ann. N.Y. Acad. Sci.*, 770:79-90, 1995.

Chen and Okayama, *Mol. Cell Biol.*, 7:2745-2752, 1987.

Chomczynski, "A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples," *Biotechniques*, 15:532-537, 1993.

Christou; Murphy; Swain, *Proc. Nat'l Acad. Sci. USA,* 84(12):3962-3966, 1987.

Coffin, *In: Virology*, Fields *et al.*, eds., Raven Press, New York, pp. 1437-1500, 1990.

Cohen, "Designing antisense oligonucleotides as pharmaceutical agents," *Trends Pharmacol. Sci.*, 10(11):435-437, 1989.

Cohen, "Antisense oligodeoxynucleotides as antiviral agents," *Antiviral Res*, 16:121-33, 1991.

Cohen, "Phosphorothioate oligodeoxynucleotides," *In: Antisense Research and Applications*, Crooke and LeBleu (Eds.), Boca Raton, FL, CRC Press, Inc. p 205-222, 1993.

Couch *et al.*, *Am. Rev. Resp. Dis.*, 88:394-403, 1963.

Coupar *et al.*, *Gene*, 68:1-10, 1988.

Cowsert, Fox, Zon, Mirabelli, "*In vitro* evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: potential treatment for genital warts," *Antimicrob. Agents Chemother*, 37:171-177, 1993.

Crooke, "An overview of progress in antisense therapeutics," *Antisense Nucleic Acid Drug Dev.*, 8:115-22, 1998b.

Crooke, Graham, Cooke, Crooke, "*In vitro* pharmacokinetics of phosphorothiate antisense oligonucleotides," *J. Pharmacol. Exp. Ther.*, 275:462-473, 1995.

De Clerq, "Targets and strategies for the antiviral chemotherapy of AIDS," *Trends Plant Sci.*, 11:198-205, 1991.

De Mesmaeker *et al.*, "Antisense oligonucleotides," *Acc. Chem. Res.*, 28:366-374, 1995.

Dean, McKay, Miraglia, Geiger, Muller, Fabbro, Bennett, "Antisense oligonucleotides as inhibitors of signal transduction: development from research tools to therapeutic agents," *Biochem. Soc. Trans.*, 24(3):623-629, 1996.

D'Halluin *et al.*, "Transgenic maize plants by tissue electroporation," *Plant Cell,* 4(12):1495-1505, 1992.

Dubensky *et al., Proc. Nat'l Acad. Sci. USA,* 81:7529-7533, 1984.

Ecker, "Strategies for invasion of RNA secondary structure," *In: Antisense Research and Applications*, Boca Raton, FL. CRC Press, Inc. p 387-400, 1993.

Ecker, Vickers, Bruice, Freier, Manoharan, Zounes, "Pseudo-half-knot binding to RNA," *Science*, 257:958-961, 1992.

Epstein, Speir, Unger, "The basis of molecular strategies for treating coronary restenosis after angioplasty," *J. Am. Coll. Cardiol.*, 23:1278-88, 1992.

Fabbro, Muller, Geiger, "Antisense oligonucleotides and their anticancer activities," *In: Antisense Research and Application*, Crooke (Ed), Springer-Verlag, Berlin, p 395-425, 1998.

Fechheimer *et al., Proc. Nat'l Acad. Sci. USA,* 84:8463-8467, 1987.

Ferkol *et al., FASEB J.*, 7:1081-1091, 1993.

Ferrari, *et al., J. Virol.*, 70:3227-3234, 1996.

Field, "Viral targets for antisense oligonucleotides: a mini review," *Antiviral Res.*, 37:67-81, 1998.

Fisher *et al., J. Virol.*, 70:520-532, 1996.

Flotte *et al., Proc. Nat'l. Acad. Sci. USA*, 90:10613-10617, 1993.

Fraley *et al., Proc. Nat'l Acad. Sci. USA,* 76:3348-3352, 1979.

Friedmann, *Science*, 244:1275-1281, 1989.

Fromm *et al.,* "Stable transformation of maize after gene transfer by electroporation," *Nature*, 319:791-793, 1986

Fromm *et al.*, *Nature,* 312:791-793, 1986.

Gallie and Walbot, "Identification of the motifs within the tobacco mosaic virus 5'-leader responsible for enhancing translation," *Nucleic Acids Res.*, 20:4631-4638, 1992.

Ghosh and Bachhawat, *In: Liver diseases, targeted diagnosis and therapy using specific receptors and ligands*, (Wu G, Wu C ed.), New York: Marcel Dekker, pp. 87-104, 1991.

Ghosh-Biswas *et al.*, "Transgenic Indica rice (*Oryza sativa L.)* plants obtained by direct gene transfer to protoplasts," *J. Biotechnol.*, 32(1):1-10, 1994.

Ghosh-Choudhury *et al.*, *EMBO J.*, 6:1733-1739, 1987.

Goelet, Lomonossoff, Butler, Akam, Gait, Karn, "Nucleotide sequence of tobacco mosaic virus RNA," *Proc. Natl. Acad. Sci. USA*, 79:5818-5822, 1982.

Gomez-Foix *et al.*, *J. Biol. Chem.*, 267:25129-25134, 1992.

Gooding Jr. and Hebert, "A simple technique for purification of tobacco mosaic virus in large quantities," *Phytopathology*, 57:1285, 1967.

Goodman, *et al.*, *Blood*, 84:1492-1500, 1994.

Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.

Gossen and Bujard, *Proc. Nat'l Acad. Sci. USA,* 89:5547-5551, 1992.

Gossen *et al., Science,* 268:1766-1769, 1995.

Graham & Prevec, *Biotechnology*, 20:363-390, 1992.

Graham and Prevec, *In: Methods in Molecular Biology: Gene Transfer and Expression Protocols 7*, E.J. Murray (ed.), Clifton, N.J., Humana Press, pp. 205-225. 1991.

Graham and Van Der Eb, *Virology*, 52:456-467, 1973.

Graham *et al.*, *J. Gen. Virol.*, 36:59-72, 1977.

Green, Pines, Inouye, "The role of antisense RNA in gene regulation," *Ann. Rev. Biochem.,* 55:569-97, 1986.

Grunhaus and Horwitz, *Seminar in Virology*, 3:237-252, 1992.

Hagio *et al.*, "Stable transformation of sorghum cell cultures after bombardment with DNA coated microprojectiles," *Plant Cell Rep.*, 10(5):260-264, 1991.

Hall and Brostoff, 1992, "Specific inhibition of IgE antibody production by an antisense oligodeoxynucleotide oligomer," *Immunology*, 77:462-4.

Harland and Weintraub, *J. Cell Biol.*, 101:1094-1099, 1985.

He *et al., Plant Cell Reports*, 14 (2-3):192-196, 1994.

Helene and Toulme, "Specific regulation of gene expression by antisense, sense and antigene nucleic acids," *Biochim. Biophys. Acta*, 1049:99-125, 1990.

Hensgens *et al.*, "Transient and stable expression of gusA fusions with rice genes in rice, barley and perennial ryegrass," *Plant Mol. Biol.*, 22(6):1101-1127, 1993.

Hermonat and Muzycska, *Proc. Nat'l Acad. Sci. USA*, 81:6466-6470, 1984.

Hersdorffer *et al.*, *DNA Cell Biol.*, 9:713-723, 1990.

Herz and Gerard, *Proc. Nat'l Acad. Sci. USA*, 90:2812-2816, 1993.

Hiei *et al.*, "Transformation of rice mediated by *Agrobacterium tumefaciens*," *Plant Mol. Biol.*, 35(1-2):205-218, 1997.

Hjalt and Wagner, "Bulged-out nucleotides in antisense RNA are required for rapid target RNA binding *in vitro* and inhibition *in vivo*," *Nucleic Acids Res.*, 23(4):580-587, 1995.

Horwich *et al.*, *J. Virol.*, 64:642-650, 1990.

Hou and Lin, *Plant Physiology*, 111:166, 1996.

Hutcherson, "Clinical antiviral activities," *In: Antisense Research and Application*, Crooke (Ed.). Berlin: Springer-Verlag, p 445-62, 1998.

Ishida *et al.*, "High efficiency transformation of maize (Zea mays L.) mediated by *Agrobacterium tumefaciens*," *Nat Biotechnol.*, 14:745-50, 1996.

Jaskulski, deRiel, Mercer, Calabretta, Baserga, "Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin," *Science*, 240(4858):1544-1546, 1988.

Joki, *et al., Human Gene Ther.*, 6:1507-1513, 1995.

Jones & Shenk, *Cell*, 13:181-188, 1978.

Kaeppler *et al.*, "Silicon carbide fiber-mediated stable transformation of plant cells," *Theor. Appl. Genet.*, 84(5-6):560-566, 1992.

Kaeppler *et al.*, *Plant Cell Reports* 9: 415-418, 1990.

Kageyama, *et al., J. Biol. Chem.*, 262(5):2345-2351, 1987.

Kaneda *et al.*, *Science*, 243:375-378, 1989.

Kaplitt *et al.*, *Methods*, 10(3): 343-350, 1996

Kaplitt *et al.*, *Nat. Genet.*, 8:148-153, 1994.

Karlsson *et al.*, *EMBO J.*, 5:2377-2385, 1986.

Kato *et al.*, *J. Biol. Chem.*, 266:3361-3364, 1991.

Kessler *et al.*, *J. Leukocyte Biol.*, 60:729-736, 1996.

Klee *et al.*, *Bio-Technology*, 3(7):637-642, 1985.

Klein *et al.*, *Nature*, 327:70-73, 1987.

Knittel *et al.*, *Plant Cell Reports,* 14(2-3):81-86, 1994.

Koeberl *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 94:1426-1431, 1997.

Lavrovsky, Chen, Roy, "Therapeutic potential and mechanism of action of oligonucleotides and ribozymes," *Biochem. Mol. Med.*, 62:11-22, 1997.

Lawson, Lee, Maimone, Abramson, Dever, Merick, Thach, "Dissociation of double-stranded polynucleotide helical structures by eukaryotic initiation factors, as revealed by a novel assay," *Biochemistry*, 28:4729-4734, 1989.

Lazzeri, "Stable transformation of barley *via* direct DNA uptake. Electroporation- and PEG- mediated protoplast transformation," *Methods Mol. Biol.*, 49:95-106, 1995.

Le Gal La Salle *et al.*, *Science*, 259:988-990, 1993.

Lee *et al.*, *Korean J. Genet.*, 11(2):65-72, 1989.

Levrero *et al.*, *Gene*, 101:195-202, 1991.

Levy, Fraenkel-Conrat, Owens, *Virology,* Englewood Cliffs, NJ: Prentice-Hall, Inc. p. 447, 1994.

Lis, "Fractionation of DNA fragments by polyethylene glycol induced precipitation," *Methods Enzymol*, 165:347-353, 1980.

Lorz *et al.*, *Mol Gen Genet*, 199:178-182, 1985.

Maas *et al.*, "Preparation and transformation of monocot protoplasts," *Methods Cell Biol.*, 50:383-399, 1995.

Mann *et al.*, *Cell*, 33:153-159, 1983.

Marcotte *et al.*, *Nature*, 335:454, 1988.

Markowitz *et al.*, *J. Virol.*, 62:1120-1124, 1988.

Matthews, *Plant Virology*, San Diego: Academic Press, Inc. p. 835, 1991.

McCabe, Martinell, *Bio-Technology*, 11(5):596-598, 1993.

McCormac *et al.*, *Euphytica*, v. 99 (1) p. 17-25 :. 1998.

McCown *et al.*, *Brain Res.*, 713:99-107, 1996.

Mirabelli and Crooke, "Antisense oligonucleotides in the context of modern molecular drug discovery and development," *In: Antisense Research and Applications*, Crooke and LeBleu (Eds.). Boca Raton, FL, CRC Press, Inc. p 7-35, 1993.

Mizukami, *et al.*, *Virology*, 217:124-130, 1996.

Mol, van der Krol, van Tunen, van Blokland, de Lange, Stuitje, "Regulation of plant gene expression by antisense RNA," *Fed. Eur. Biochem. Soc.*, 268(2):427-30, 1990.

Mulligan, *Science*, 260:926-932, 1993.

Murray, editor, Antisense RNA and DNA, New York: Alan R. Liss, 1992.

Nagatani *et al.*, "DNA delivery into rice cells and transformation using silicon carbide whiskers," *Biotech. Tech.*, 11(7):471-473, 1997.

Needleman and Wunsch, *J. Mol. Biol.*, 48:443, 1970, as revised by Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981.

Nejidat and Beachy, "Transgenic tobacco plants expressing a coat protein gene of tobacco mosaic virus are resistant to some other tobamoviruses," *Mol. Plant Microbe. Interact.* 3:247-251, 1990.

Nicolas and Rubenstein, *In: Vectors: A survey of molecular cloning vectors and their uses*, Rodriguez and Denhardt (eds.), Stoneham: Butterworth, pp. 493-513, 1988.

Nicolau and Sene, *Biochim. Biophys. Acta*, 721:185-190, 1982.

Nicolau *et al.*, *Methods Enzymol.*, 149:157-176, 1987.

Nietfeld, Duits, Tilanus, "Antisense oligonucleotides, a novel tool for the control of cytokine effects on human cartilage," *Arthritis Rheum.*, 37:1357-1362, 1994.

Olivierio, *et al., EMBO J.*, 6(7):1905-1912, 1987.

Omirulleh *et al.*, "Activity of a chimeric promoter with the doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize." *Plant Mol. Biol.*, 21:415-28, 1993.

Ooms, Hooykaas, van Veen, van Beelen, Regenburg-tuink, Shilperoort, "Octopine Ti-plasmid deletion mutants of Agrobacterium tumefaciens with emphasis on the right side of the T-region," *Plasmid*, 7:15-29, 1982.

Paskind *et al.*, *Virology*, 67:242-248, 1975.

Perales *et al.*, *Proc. Nat'l Acad. Sci.* 91:4086-4090, 1994.

Peris, Jung, Resnick, Walker, Malakhova, Bokrand, Wielbo, "Antisense inhibition of striatal $GABA_A$ receptor proteins decreases GABA-stimulated chloride uptake and increases cocaine sensitivity in rats," *Mol. Brain Res.*, 57:310-312, 1998.Abdullah *et al.*, *Biotechnology*, 4:1087, 1986.

Ping, *et al., Microcirculation*, 3:225-228, 1996.

Poli and Cortese, *Proc. Natl. Acad. Sci. USA*, 86:8202-8206, 1989.

Ponnazhagan, *et al.*, *Gene*, 190:203-210, 1997c.

Ponnazhagan, *et al.*, *Hum. Gene Ther.*, 8:275-284, 1997a.

Ponnazhagan, *et al.*, *J. Virol.*, 71:,1997b.

Potrykus *et al.*, *Mol. Gen. Genet.*, 199:183-188, 1985.

Potter *et al.*, *Proc. Nat'l Acad. Sci. USA*, 81:7161-7165, 1984.

Prowse and Baumann, *Mol Cell Biol,* 8(1):42-51, 1988.

Racher *et al.*, *Biotechnology Techniques*, 9:169-174, 1995.

Ragot *et al.*, *Nature*, 361:647-650, 1993.

Rhodes *et al.*, "Transformation of maize by electroporation of embryos," *Methods Mol. Biol.,* 55:121-131, 1995.

Rich *et al.*, *Hum. Gene Ther.*, 4:461-476, 1993.

Ridgeway, *In: Vectors: A survey of molecular cloning vectors and their uses*, Rodriguez RL, Denhardt DT, ed., Stoneham: Butterworth, pp. 467-492, 1988.

Rippe *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.

Ritala *et al.*, "Fertile transgenic barley to particle bombardment of immature embryos," *Plant Mol. Biol.*, 24(2):317-325, 1994.

Rogers *et al.*, *Methods Enzymol.*, 153:253-277, 1987.

Rogers, Horsch, Fraley, "Gene transfer in plants: production of transformed plants using Ti plasmid vectors," *Methods Enzymol*, 118:627-640, 1986.

Ron, *et al., Mol. Cell. Biol.*, 2887-2895, 1991.

Rosenfeld *et al.*, *Cell*, 68:143-155, 1992.

Rosenfeld *et al.*, *Science*, 252:431-434, 1991.

Roux *et al.*, *Proc. Nat'l Acad. Sci. USA*, 86:9079-9083, 1989.

Shanahan Jr., "Properties of ISIS 2302, an inhibitor of intercellular adhesion molecule-1, in humans," *In: Antisense Research and Application*, Crooke (Ed), Berlin: Springer-Verlag, p 499-524, 1998.

Shimoni *et al.*, "A recombinant protein of two high molecular weight glutenins alters gluten polymer formation in transgenic wheat," *J. Biol. Chem.*, 272:15488-15495, 1997.

Simons, "The control of prokaryotic and eukaryotic gene expression by naturally occurring antisense RNA," *In: Antisense Research and Applications*, Crooke and Lebleu (Eds.), Boca Raton, FL, CRC Press, Inc. p 97-124, 1993.

Singsit *et al.*, "Expression of a Bacillus thuringiensis cryIA(c) gene in transgenic peanut plants and its efficacy against lesser cornstalk borer," *Transgenic Res.*, 6:169-76, 1997.

Stratford-Perricaudet and Perricaudet, *In: Human Gene Transfer*, O. Cohen-Haguenauer *et al.*, eds., John Libbey Eurotext, France, pp. 51-61, 1991.

Stratford-Perricaudet *et al.*, *Hum. Gene. Ther.*, 1:241-256, 1990.

Svab, Hajdukiewicz, Maliga, "Generation of transgenic tobacco plants by cocultivation of leaf disks with pPZP Agrobacterium binary vectors," *In: Methods in Plant Molecular Biology -- A Laboratory Manual*, Maliga, Klessig, Cashmore, Gruissem, Varner (Eds.), Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p 55-77, 1995.

Takamatsu, Watanabe, Iwasaki, Shiba, Meshi, Okada, "Deletion analysis of the 5' untranslated leader sequence of tobacco mosaic virus RNA," *J. Virol.*, 65(3):1619-1622, 1991.

Takumi and Shimada, "Variation in transformation frequencies among six common wheat cultivars through particle bombardment of scutellar tissues," *Genes Genet. Sys.*, 72:63-69, 1997.

Temin, *In: Gene Transfer*, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986.

Thompson *et al.*, "Maize transformation utilizing silicon carbide whiskers: A review," *Euphytica*, 85(1-3):75-80, 1995.

Tingay *et al.*, *The Plant Journal* v. 11 (6) p. 1369-1376. 1997.

Tomes *et al.*, "Transgenic tobacco plants and their progeny derived by microprojectile bombardment of tobacco leaves," *Plant Mol. Biol.*, 14:261-8, 1990

Top *et al.*, *J. Infect. Dis.*, 124:155-160, 1971.

Torbet *et al.*, "Transformation of oat using mature embryo-derived tissue cultures," *Crop Science*, 38:226-231, 1998.

Torbet *et al.*, "Use of paromomycin as a selective agent for oat transformation," *Plant Cell Reports*, 14:635-640, 1995.

Toriyama *et al.*, *Theor Appl. Genet.*, 73:16, 1986.

Tsukada; Kusano; Kitagawa, *Plant Cell Physiol.*, 30(4)599-604, 1989.

Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.

Uchimiya *et al.*, *Mol. Gen. Genet.*, 204:204, 1986.

Uhlmann and Peyman, "Antisense oligonucleotides: a new therapeutic principle," *Chem. Rev.*, 90(4):543-84, 1990.

Van Eck; Blowers; Earle, *Plant Cell Reports*, 14(5):299-304, 1995.

Varmus *et al.*, *Cell*, 25:23-36, 1981.

Vasanthakumar and Ahmed, "Modulation of drug resistance in a daunorubicin resistant subline with oligonucleoside methylphosphonates," *Cancer Commun.*, 1(4):225-232, 1989.

Vasil *et al.*, *Plant Physiol.*, 91:1575-1579, 1989.

Wagner *et al., Proc. Nat'l Acad. Sci.* 87(9):3410-3414, 1990.

Walther and Stein, *J. Mol. Med*, 74:379-392, 1996.

Weiss, Davidkova, Zhou, "Antisense RNA gene therapy for studying and modulating biological processes," *Cell Mol. Life Sci.*, 55(3):334-358, 1999.

Wilson, *et al.*, *Mol. Cell. Biol.*, 6181-6191, 1990.

Wong *et al., Gene,* 10:87-94, 1980.

Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.

Wu and Wu, *Biochemistry*, 27:887-892, 1988.

Wu and Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.

Xiao, *et al.*, *J. Virol.*, 70:8098-8108, 1996.

Yacyshyn, Woloschuk, Yacyshyn, Martini, Doan, Tami, Bennett, Kisner, Shanahan, "Efficacy and safety of ISIS 2302 (ICAM-1 antisense oligonucleotide) treatment of steroid-dependent Crohn's disease," *Gastroenterology*, 112:A1123, 1997.

Yamada *et al.*, *Plant Cell Rep.*, 4:85, 1986.

Yang *et al., Proc. Nat'l Acad. Sci USA,* 87:9568-9572, 1990.

Zechner *et al., Mol. Cell. Biol.*, 2394-2401, 1988.

Zelenin *et al.*, *FEBS Lett.*, 280:94-96, 1991.

Zhang *et al.*, "Agrobacterium-mediated transformation of elite indica and japonica rice cultivars," *Mol. Biotechnol.*, 8(3):223-231, 1997.

Zheng and Edwards, "Expression of resistance to barley stripe mosaic virus in barley and oat protoplasts," *J. Gen. Virol.*, 71:1865-1868, 1990.

Zhou *et al., Plant Cell Reports,* 12(11).612-616, 1993.

CLAIMS:

1. A method comprising contacting a target nucleic acid with an antisense nucleic acid comprising sequences complementary to a first region in the target nucleic acid and a second region in the target nucleic acid , wherein the regions are not contiguous in the target nucleic acid.

2. The method of claim 1, where in the antisense nucleic acid is further defined as comprising a first sequence complementary to the first region of the target nucleic acid and a second sequence complementary to the second region of the target nucleic acid .

3. The method of claim 2, wherein the first sequence and the second sequence are contiguous in the antisense nucleic acid.

4. The method of claim 2, wherein the first sequence and second sequence are not contiguous in the antisense nucleic acid.

5. The method of claim 1, wherein the target nucleic acid is mRNA.

6. The method of claim 1, further defined as a method of inhibiting transcription of mRNA.

7. The method of claim 1, wherein the nucleic acid is in a cell.

8. The method of claim 7, wherein the cell is in an animal.

9. The method of claim 7, wherein the cell is in a plant.

10. The method of claim 7, wherein the cell is in a human.

11. The method of claim 1, wherein the antisense nucleic acid is about 20 bases.

12. The method of claim 1, wherein the antisense nucleic acid is about 30 bases.

13. The method of claim 1, wherein the antisense nucleic acid is about 50 bases.

14. The method of claim 1, wherein the antisense nucleic acid is comprised in an expression vector.

15. The method of claim 1, further defined as a method of protecting a cell from pathogen attack, wherein the target nucleic acid is a pathogen nucleic acid.

16. The method of claim 15, wherein the pathogen is a virus.

17. The method of claim 16, wherein the cell is a plant cell.

18. The method of claim 15 wherein the protecting comprises inhibiting viral replication.

19. The method of claim 1, further defined as a method of treating a disease state associated with expression of a selected gene product, wherein the target nucleic acid is an mRNA encoding the selected gene product.

20. An antisense nucleic acid comprising sequences complementary to a first region in a target nucleic acid and a second region in the target nucleic acid, wherein the regions are not contiguous in the target nucleic acid.

21. The antisense nucleic acid of claim 20, further defined as comprising a first sequence complementary to the first region of the target nucleic acid and a second sequence complementary to the second region of the target nucleic acid.

22. The antisense nucleic acid of claim 21, wherein the first sequence and the second sequence are contiguous in the antisense nucleic acid.

23. The antisense nucleic acid of claim 21, wherein the first sequence and second sequence are not contiguous in the antisense nucleic acid.

24. The antisense nucleic acid of claim 23, wherein the first sequence and second sequence are linked by a linker.

25. The antisense nucleic acid of claim 24, wherein the linker is a nucleic acid segment.

26. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a 5' non-translated region in the target nucleic acid.

27. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to an AUG in the target nucleic acid.

28. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a translation initiation factor binding sequence in the target nucleic acid.

29. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a ribosome subunit binding sequence in the target nucleic acid.

30. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a Shine Dalgarno sequence in the target nucleic acid.

31. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a 3' non-translated sequence in the target nucleic acid.

32. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a poly-addition site in the target nucleic acid.

33. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a 3' mRNA cleavage site in the target nucleic acid.

34. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to a coding region in the target nucleic acid.

35. The antisense nucleic acid of claim 20, wherein one or both of the regions is complementary to an intron, intron branch, intron/exon junction, or splice sequence in the target nucleic acid.

36. The antisense nucleic acid of claim 20, wherein the target nucleic acid encodes an oncogene, angiogenic gene, tumor suppressor, inducers of apoptosis, enzyme, transcription factor regulator, cell cycle regulator, viral sequence, or and bacterial sequence.

37. The antisense nucleic acid of claim 20, wherein the antisense nucleic acid is about 20 bases.

38. The antisense nucleic acid of claim 20, wherein the antisense nucleic acid is about 30 bases.

39. The antisense nucleic acid of claim 20, wherein the antisense nucleic acid is about 50 bases.

40. The antisense nucleic acid of claim 20, wherein the antisense nucleic acid is about 100 bases.

41. The antisense nucleic acid of claim 20, wherein the antisense nucleic acid is about 500 bases.

42. The antisense nucleic acid of claim 20, wherein the antisense nucleic acid is about 1000 bases.

43. A pharmaceutical composition comprising an antisense nucleic acid comprising sequences complementary to two regions in a target nucleic acid, wherein

the regions are not contiguous in the target nucleic acid, dispersed in a pharmacologically acceptable buffer, diluent or excipient.

44. The pharmaceutical composition of claim 43, wherein the antisense nucleic acid is comprised in an expression vector.

45. A cell comprising an antisense nucleic acid comprising sequences complementary to two regions in a target nucleic acid, wherein the regions are not contiguous in the target nucleic acid.

46. The cell of claim 45, further defined as a eukaryotic cell.

47. The cell of claim 45, further defined as an animal cell.

48. The cell of claim 45, further defined as a plant cell.

49. The cell of claim 45, further defined as being comprised in a plant.

50. The cell of claim 45, further defined as being comprised in an animal.

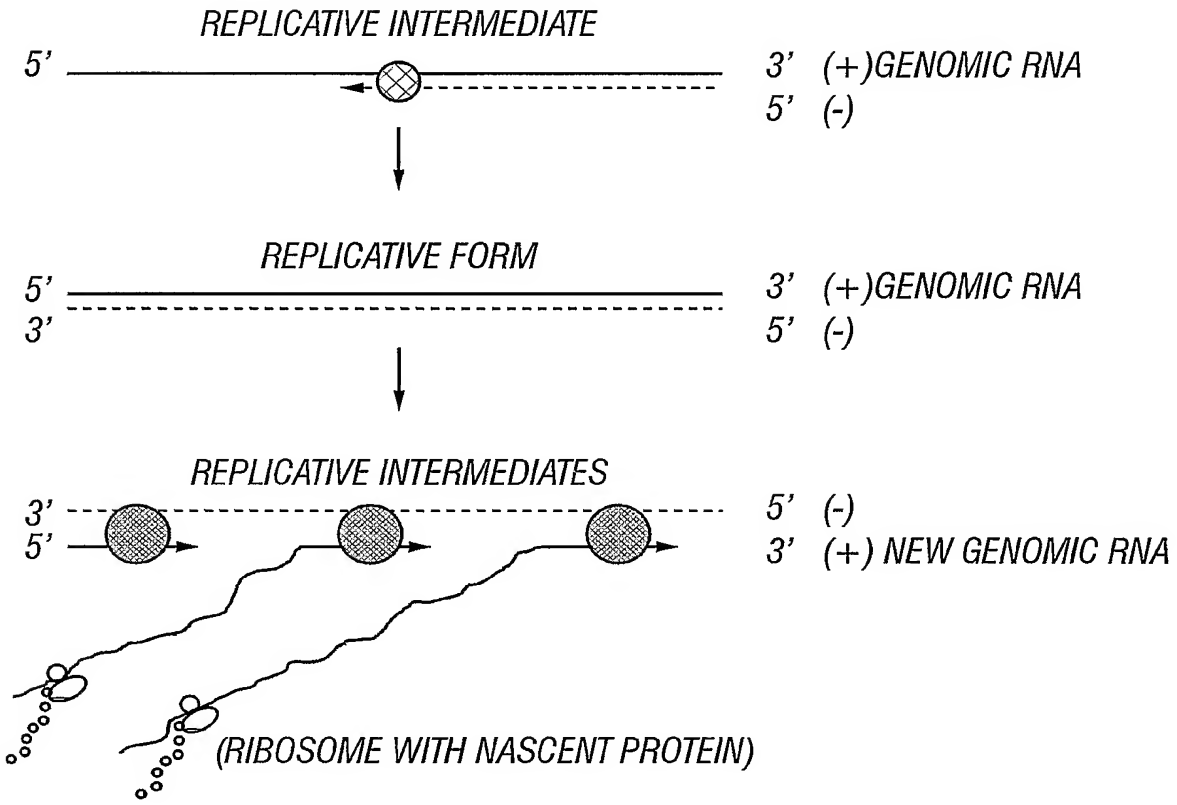
REPLICATIVE INTERMEDIATE
5'
3' (+)GENOMIC RNA
5' (-)
REPLICATIVE FORM
5'
3'
3' (+)GENOMIC RNA
5' (-)
REPLICATIVE INTERMEDIATES
3'
5'
5' (-)
3' (+) NEW GENOMIC RNA
(RIBOSOME WITH NASCENT PROTEIN)

FIG. 1

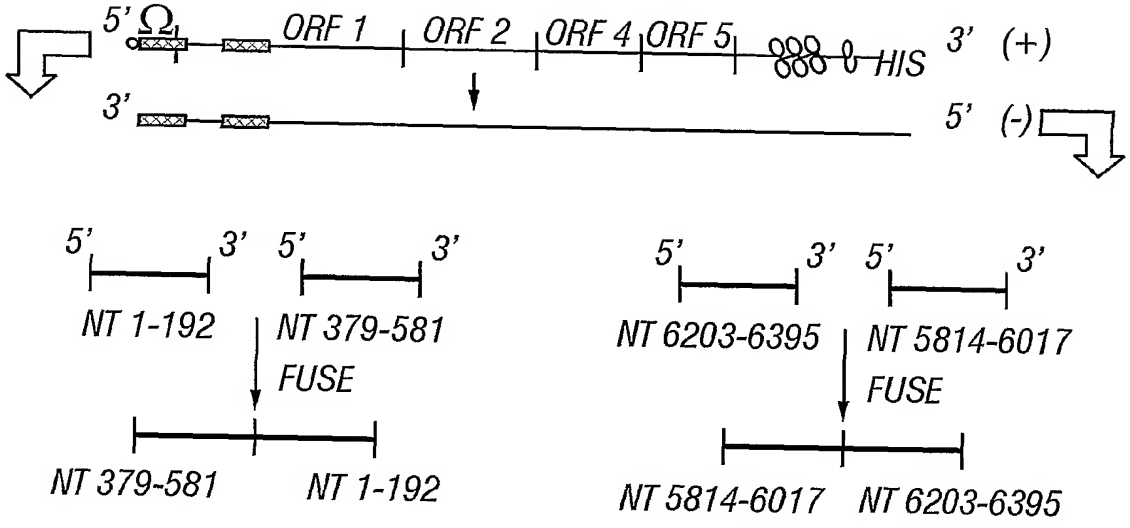
5' Ω
ORF 1
ORF 2
ORF 4
ORF 5
HIS
3' (+)
3'
5' (-)
5' 3' 5' 3'
NT 1-192
NT 379-581
FUSE
NT 379-581
NT 1-192
5' 3' 5' 3'
NT 6203-6395
NT 5814-6017
FUSE
NT 5814-6017
NT 6203-6395

FIG. 2

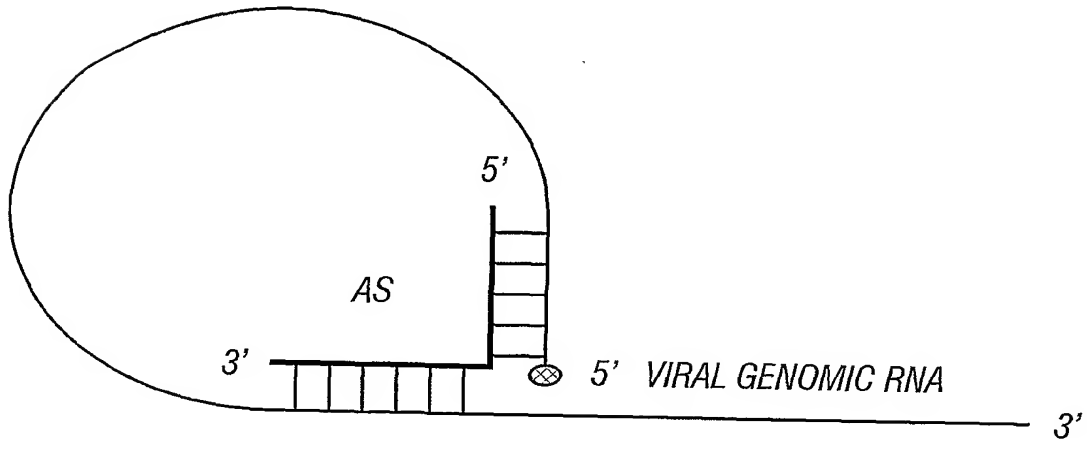
5'
AS
3'
5' VIRAL GENOMIC RNA
3'

FIG. 3

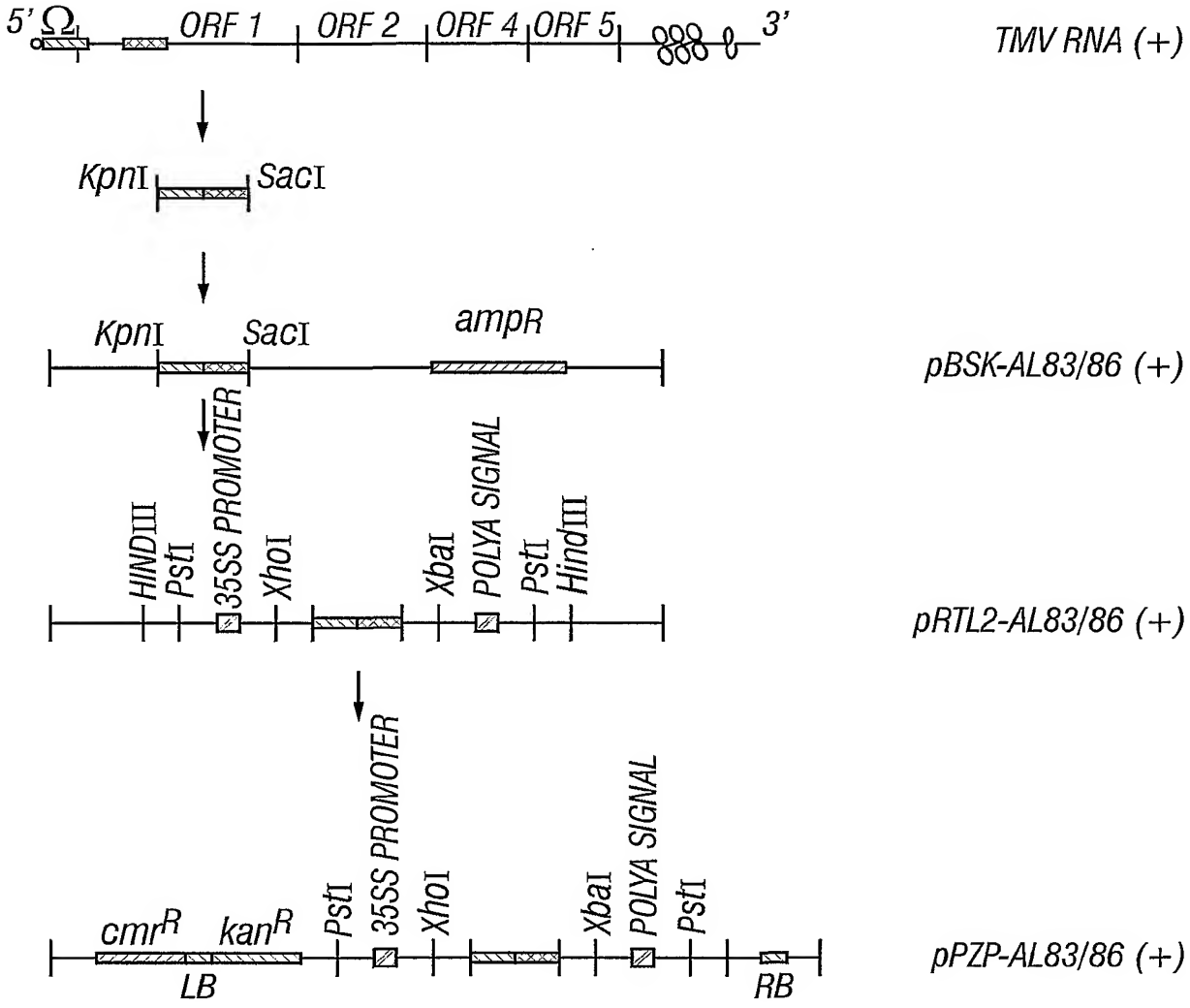
5' Ω
ORF 1
ORF 2
ORF 4
ORF 5
3'
TMV RNA (+)
KpnI
SacI
KpnI
SacI
ampR
pBSK-AL83/86 (+)
HINDIII
PstI
35SS PROMOTER
XhoI
XbaI
POLYA SIGNAL
PstI
HindIII
pRTL2-AL83/86 (+)
cmr^R
kan^R
PstI
35SS PROMOTER
XhoI
XbaI
POLYA SIGNAL
PstI
LB
RB
pPZP-AL83/86 (+)

FIG. 4A

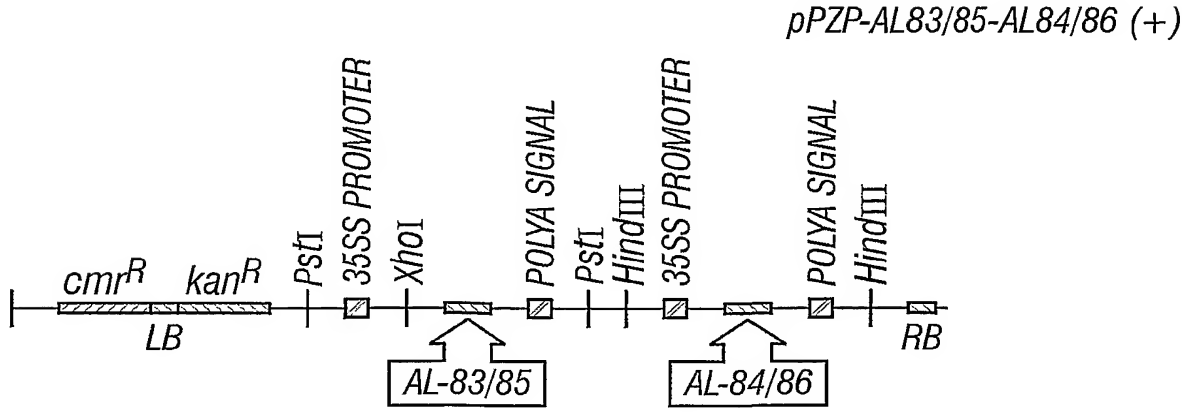
pPZP-AL83/85-AL84/86 (+)
cmr^R
kan^R
PstI
35SS PROMOTER
XhoI
POLYA SIGNAL
PstI
HindIII
35SS PROMOTER
POLYA SIGNAL
HindIII
LB
RB
AL-83/85
AL-84/86

FIG. 4B

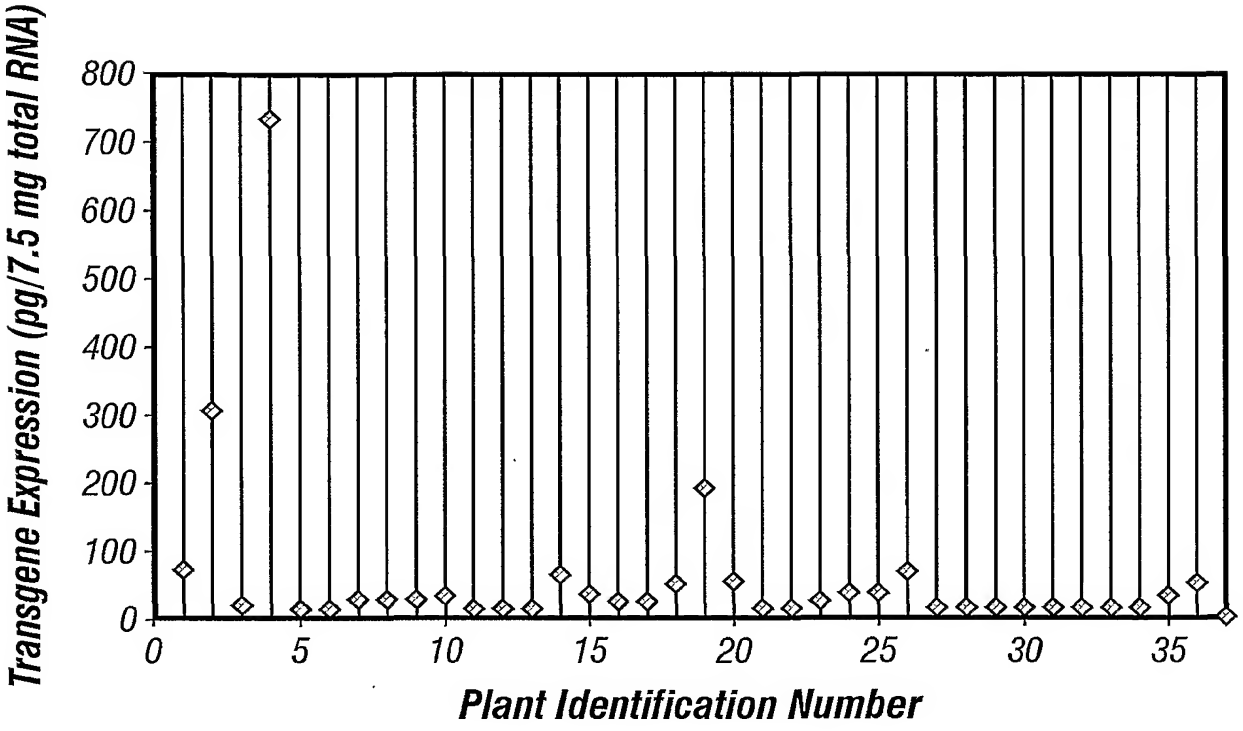
Transgene Expression (pg/7.5 mg total RNA)
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
Plant Identification Number

FIG. 5

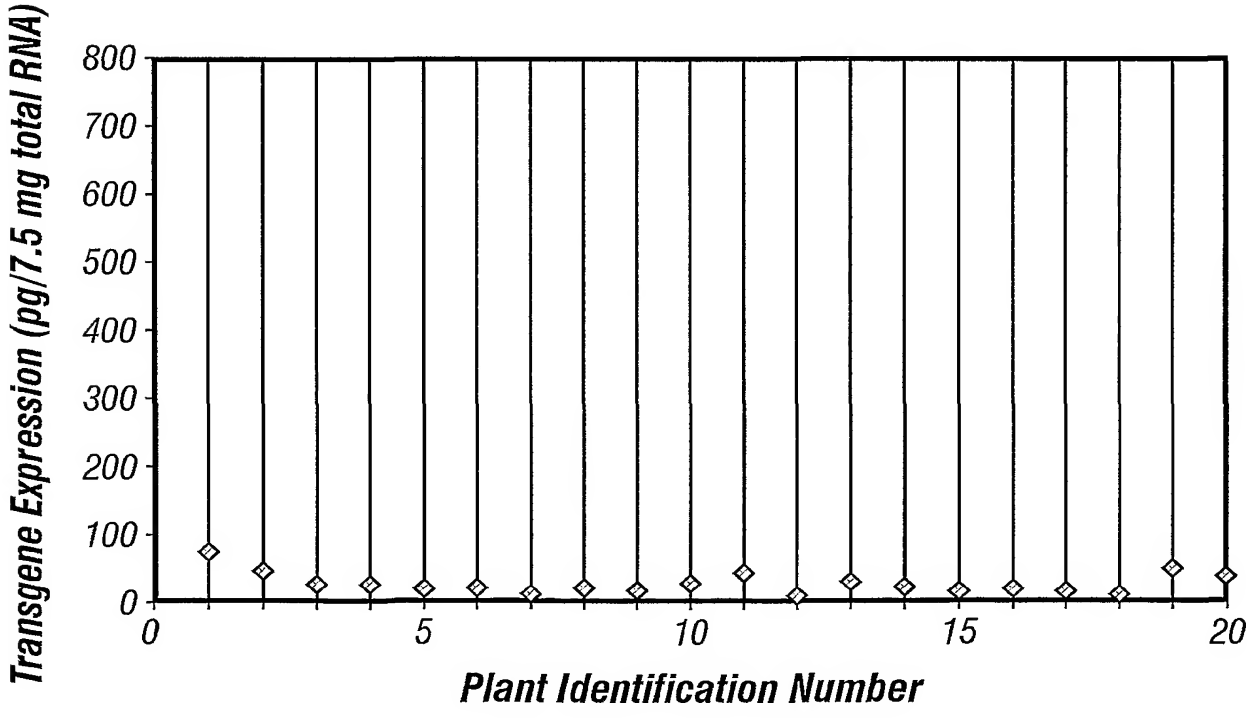
Transgene Expression (pg/7.5 mg total RNA)
800
700
600
500
400
300
200
100
0
0
5
10
15
20
Plant Identification Number

FIG. 6

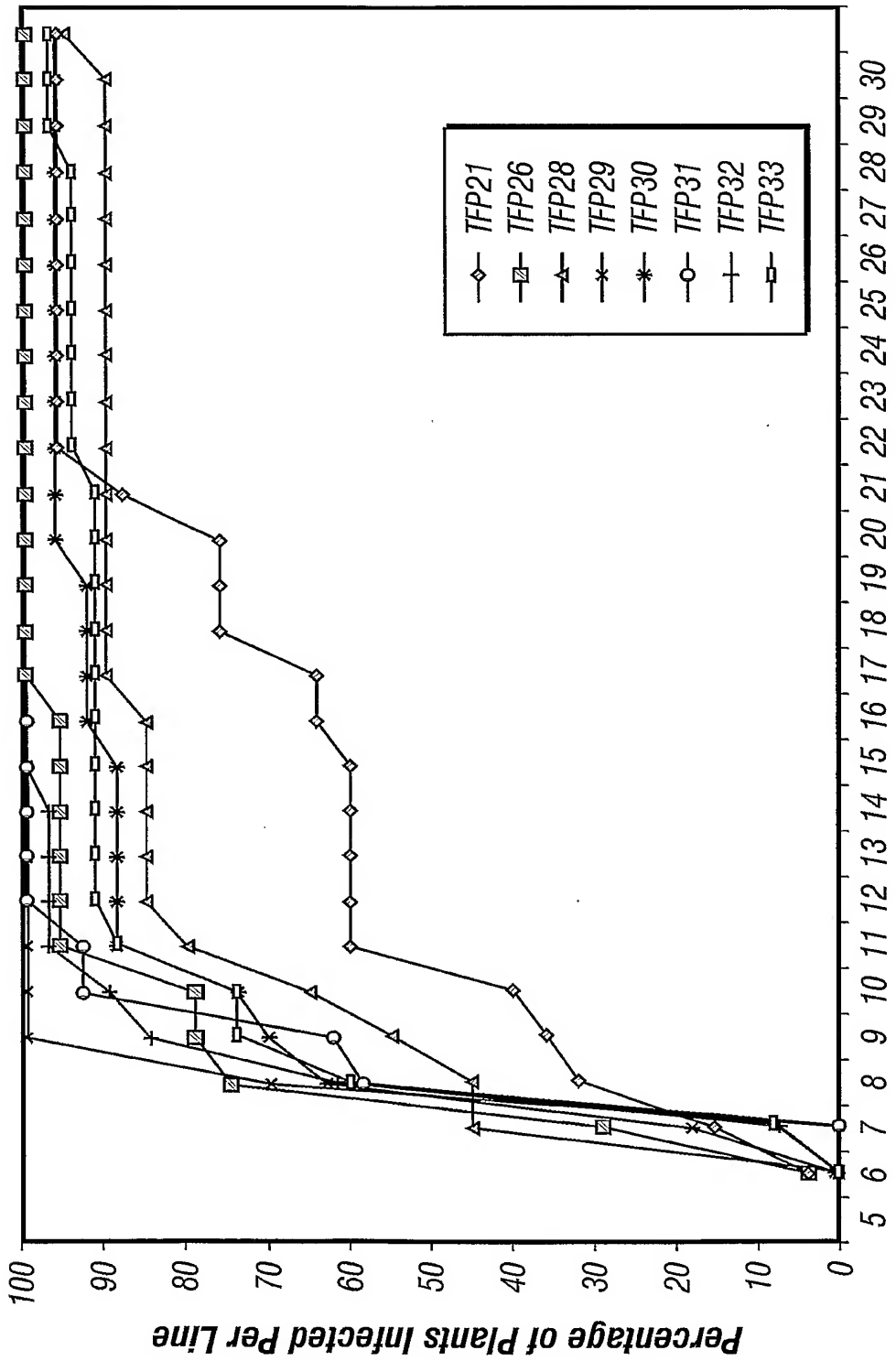
Percentage of Plants Infected Per Line
0
10
20
30
40
50
60
70
80
90
100
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Days Post Inoculation
TFP21
TFP26
TFP28
TFP29
TFP30
TFP31
TFP32
TFP33

FIG. 7

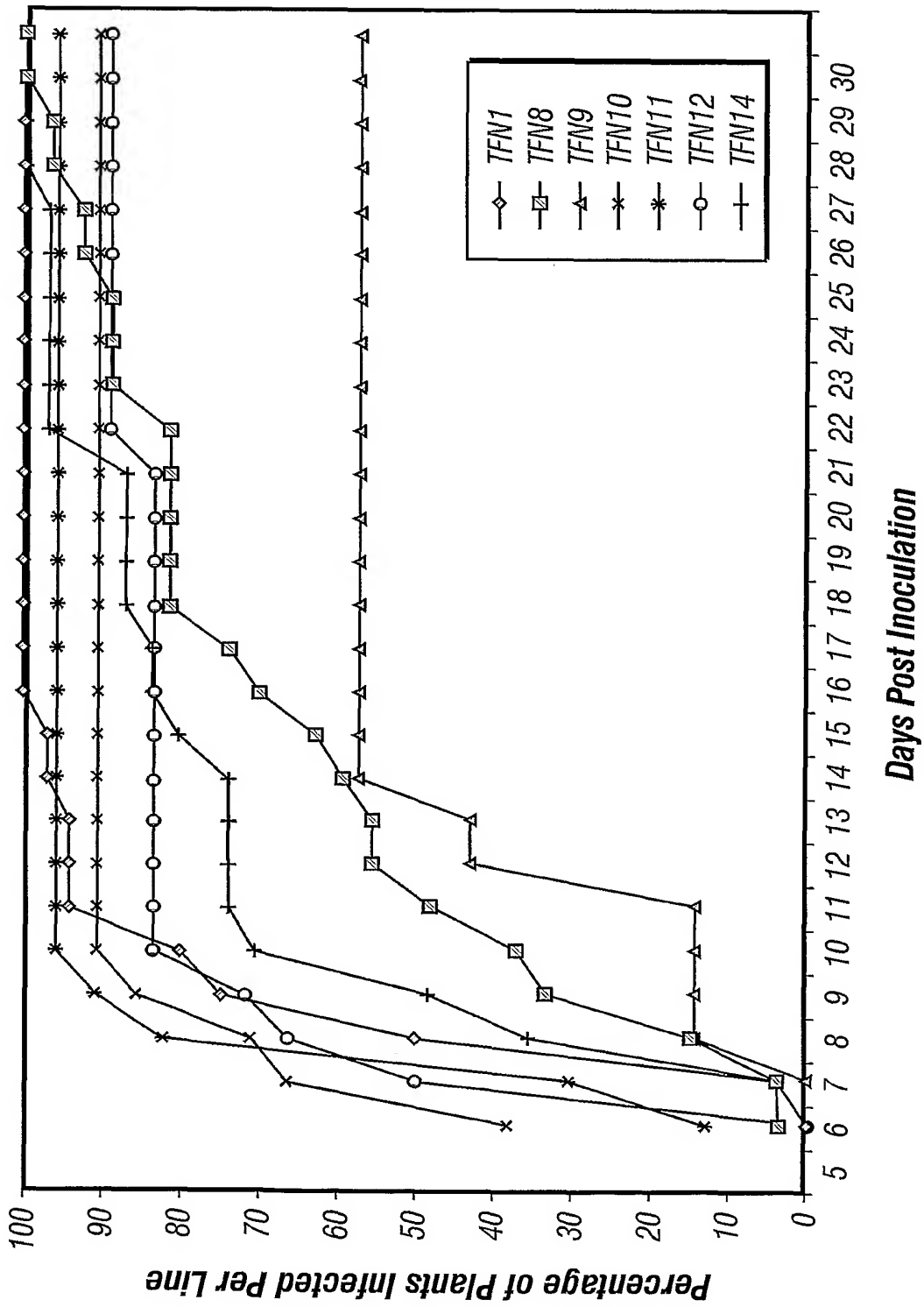
Percentage of Plants Infected Per Line
0
10
20
30
40
50
60
70
80
90
100
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Days Post Inoculation
TFN1
TFN8
TFN9
TFN10
TFN11
TFN12
TFN14

FIG. 8

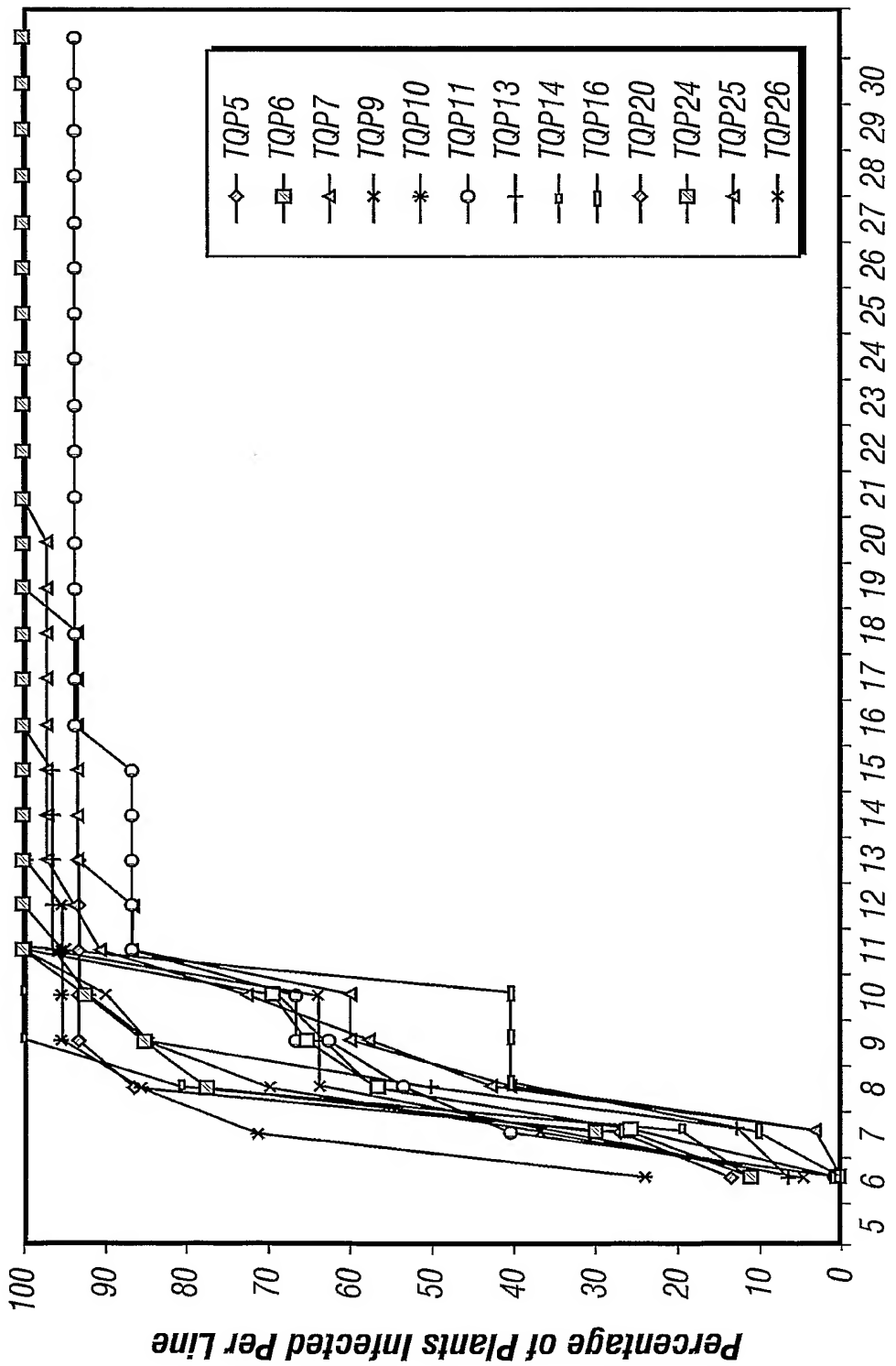
Percentage of Plants Infected Per Line
0
10
20
30
40
50
60
70
80
90
100
Days Post Inoculation
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
TQP5
TQP6
TQP7
TQP9
TQP10
TQP11
TQP13
TQP14
TQP16
TQP20
TQP24
TQP25
TQP26

FIG. 9

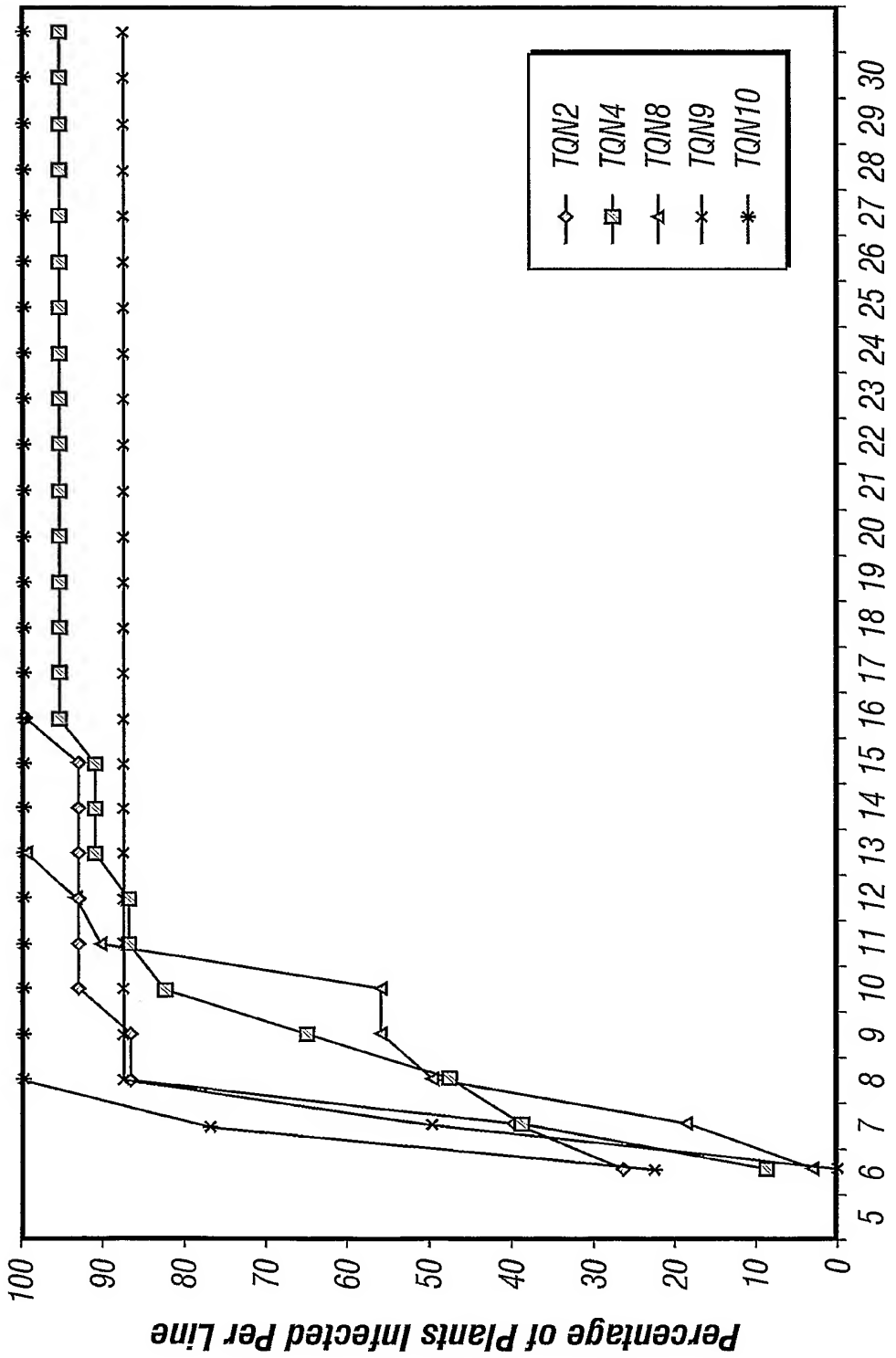
Percentage of Plants Infected Per Line
0
10
20
30
40
50
60
70
80
90
100
Days Post Inoculation
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
TQN2
TQN4
TQN8
TQN9
TQN10

FIG. 10

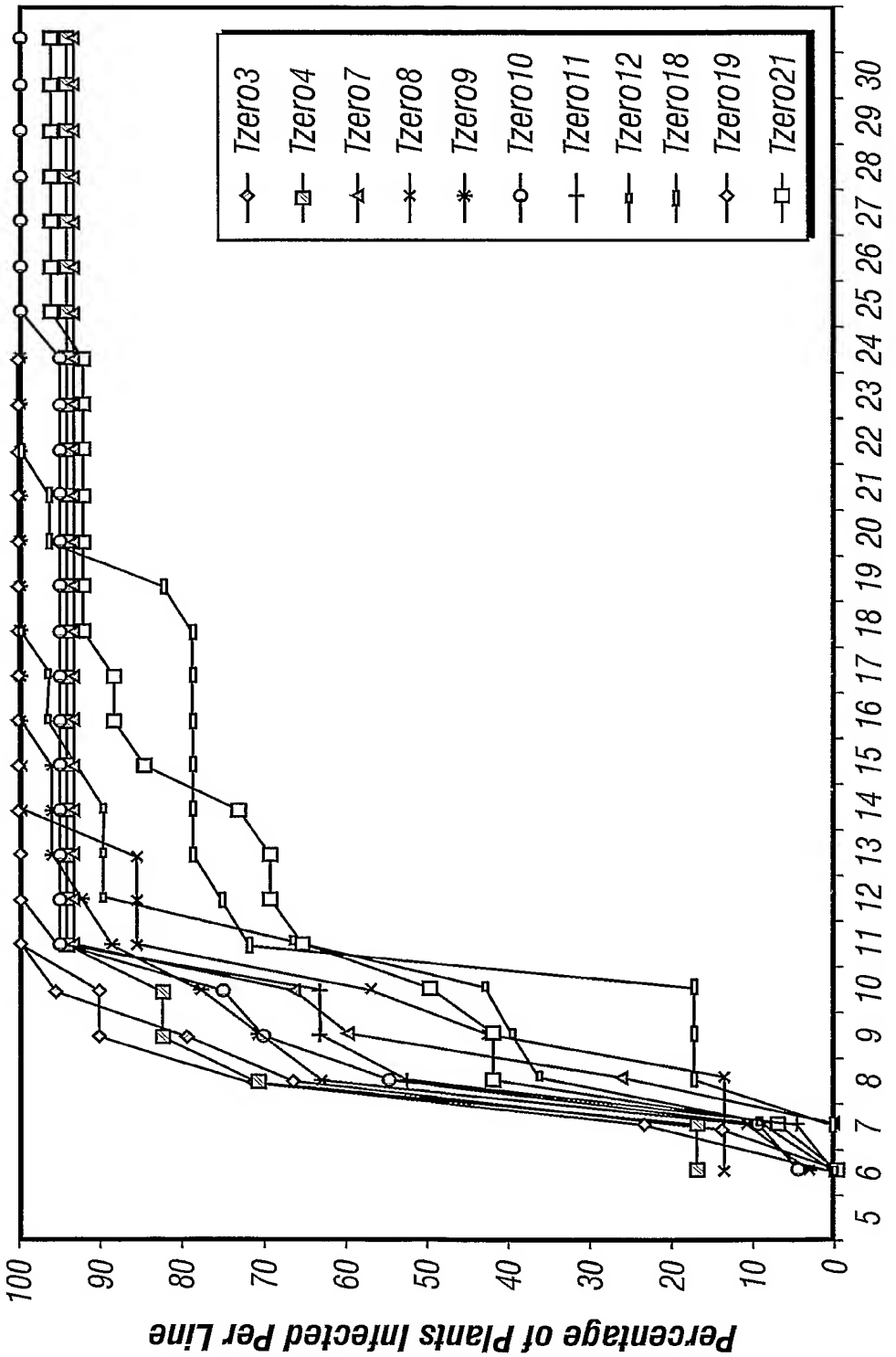
Tzero3
Tzero4
Tzero7
Tzero8
Tzero9
Tzero10
Tzero11
Tzero12
Tzero18
Tzero19
Tzero21
Percentage of Plants Infected Per Line
0
10
20
30
40
50
60
70
80
90
100
Days Post Inoculation
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

FIG. 11

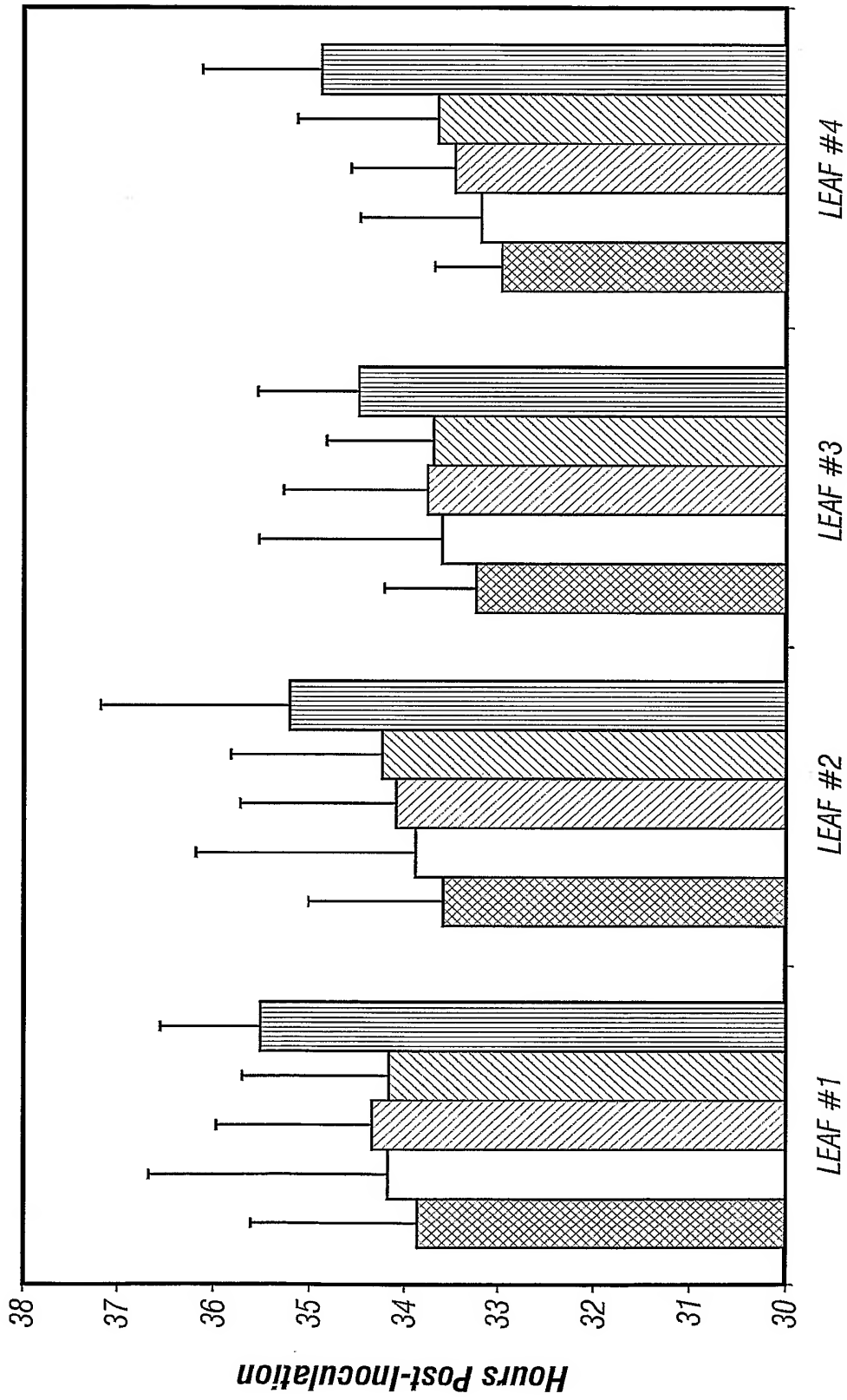
Hours Post-Inoculation
38
37
36
35
34
33
32
31
30
LEAF #1
LEAF #2
LEAF #3
LEAF #4
Leaf Position

FIG. 12

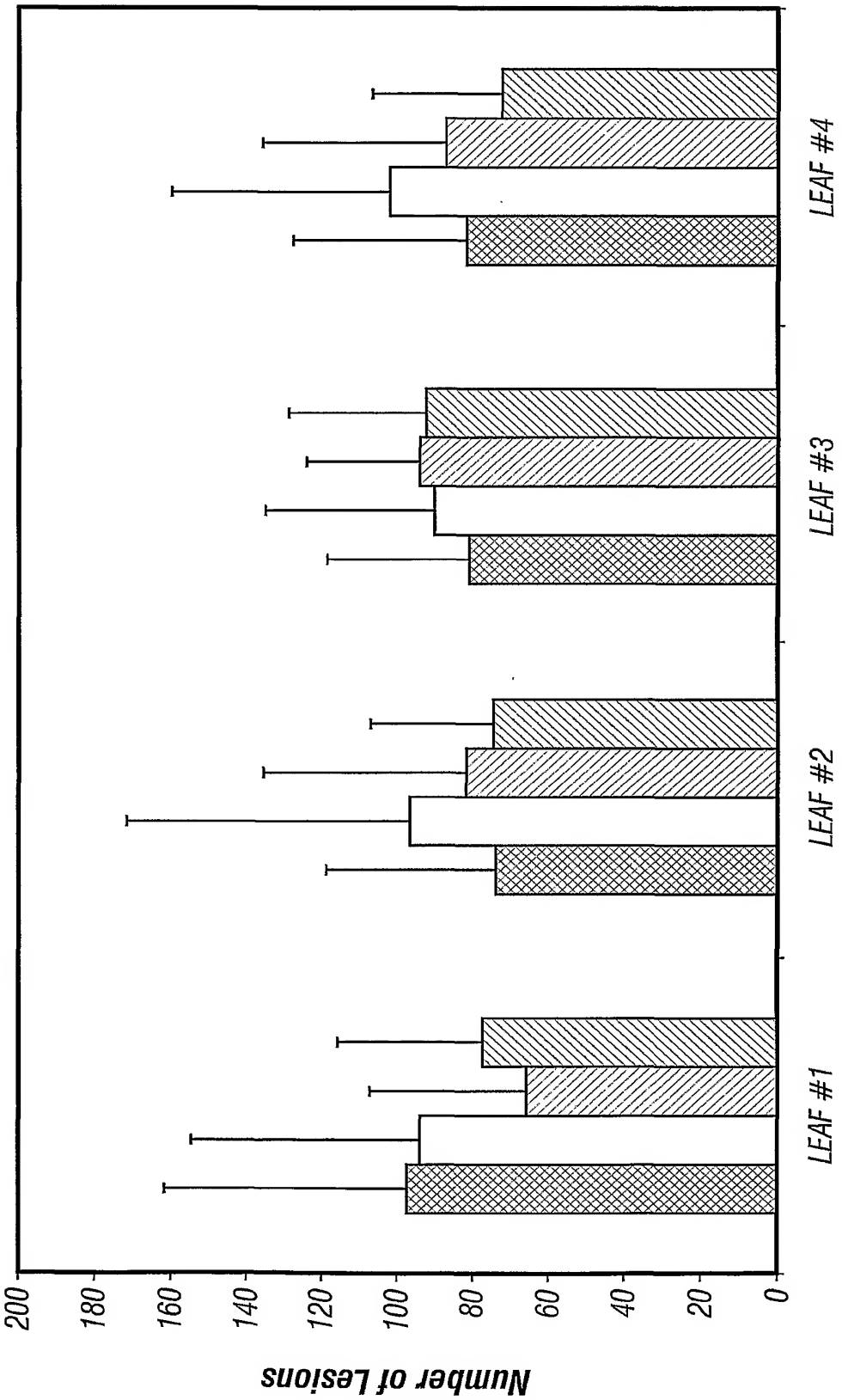
Number of Lesions
200
180
160
140
120
100
80
60
40
20
0
LEAF #1
LEAF #2
LEAF #3
LEAF #4
Leaf Position

FIG. 13

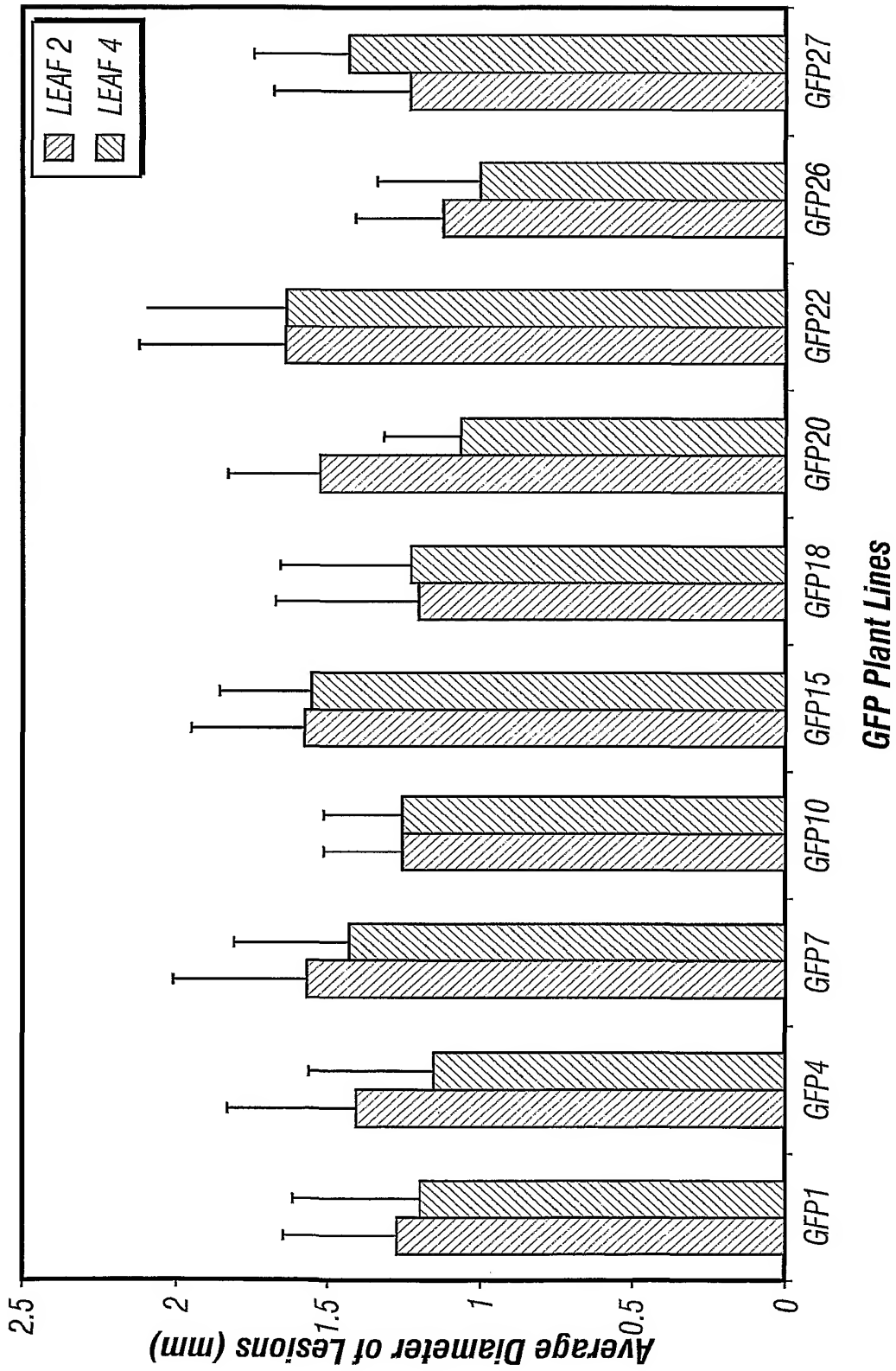
LEAF 2
LEAF 4
Average Diameter of Lesions (mm)
2.5
2
1.5
1
0.5
0
GFP1
GFP4
GFP7
GFP10
GFP15
GFP18
GFP20
GFP22
GFP26
GFP27
GFP Plant Lines

FIG. 14

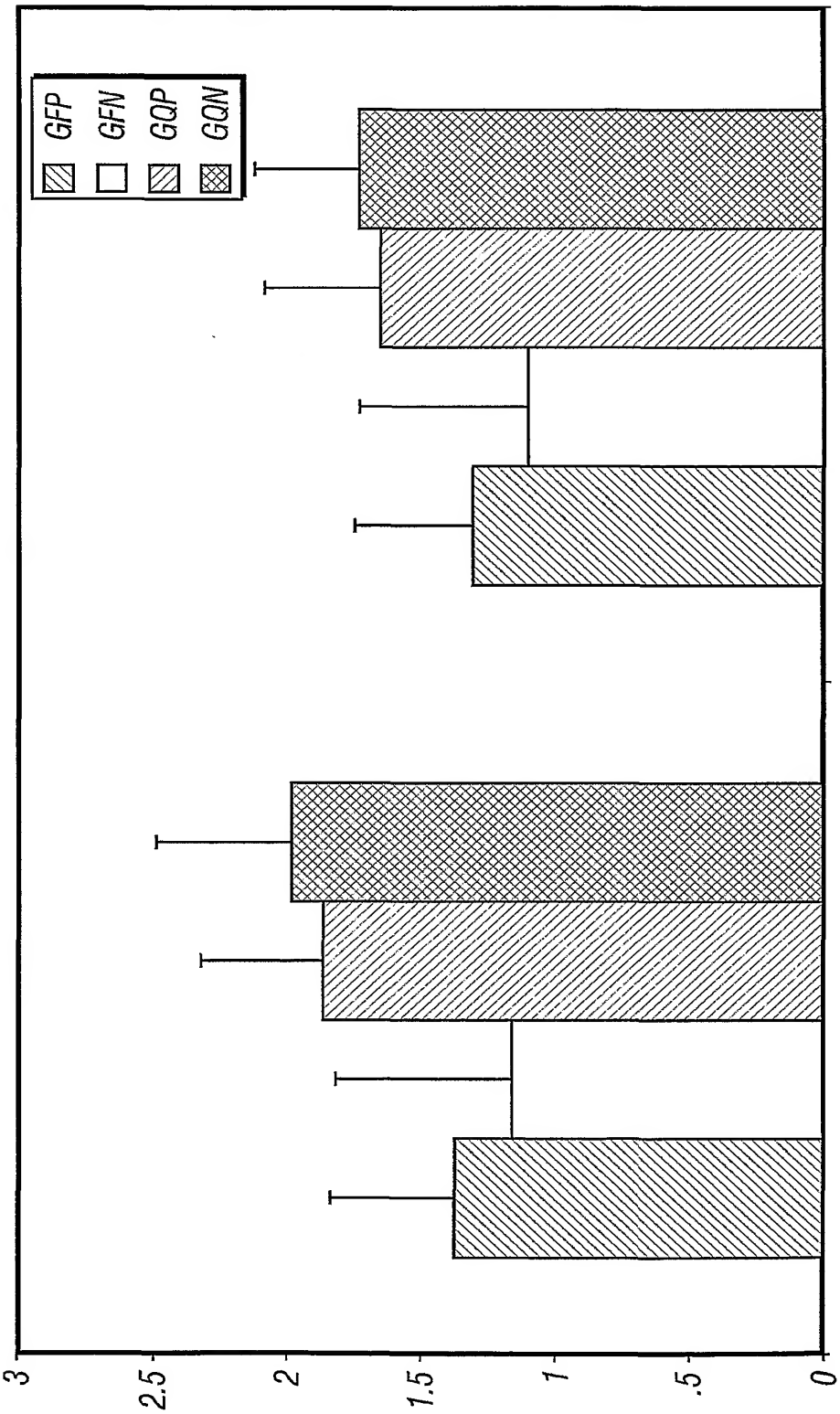

FIG. 15

INTERNATIONAL SEARCH REPORT

Int[illegible]nal Application No
PCT/US 01/00754

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 A61K31/7088 C12N5/10 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, MEDLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ENGDAHL HILDE M ET AL: "A two unit antisense RNA cassette test system for silencing of target genes." NUCLEIC ACIDS RESEARCH, vol. 25, no. 16, 1997, pages 3218-3227, XP002168309 ISSN: 0305-1048	1-3,5-7, 14, 20-22, 26,29, 30,34, 36,45
Y	the whole document	1-50
Y	EP 0 331 939 A (GREATBATCH GEN AID LTD) 13 September 1989 (1989-09-13) column 8, line 39 -column 11, line 42 claims; examples	1-50

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 2001	11/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CLOAD S T ET AL: "KINETIC AND THERMODYNAMIC ANALYSIS OF RNA BINDING BY TETHERED OLIGONUCLEOTIDE PROBES: ALTERNATIVE STRUCTURES AND CONFORMATIONAL CHANGES" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 115, no. 12, 1993, pages 5005-5014, XP000910296 ISSN: 0002-7863 the whole document	1,2,4, 20,21, 23,24
X	FRANCOIS JEAN-CHRISTOPHE ET AL: "Recognition and cleavage of hairpin structures in nucleic acids by oligodeoxynucleotides." NUCLEIC ACIDS RESEARCH, vol. 22, no. 19, 1994, pages 3943-3950, XP002168310 ISSN: 0305-1048 the whole document	1-3,5,6, 20-22
X	FRANCOIS J C ET AL.: "Recognition of hairpin-containing single-stranded DNA by oligonucleotides containing internal acridine derivatives" BIOCONJUGATE CHEMISTRY, (MAY-JUN 1999) VOL. 10, NO. 3, PP. 439-446., XP002168311 the whole document	1,2,4, 11,20, 21, 23-25,37
X	US 5 399 676 A (FROEHLER BRIAN) 21 March 1995 (1995-03-21) column 13, line 29 -column 15, last line claims	1,2,4,7, 20,21, 23,24, 43,45
A	WO 97 38097 A (HYBRIDON INC) 16 October 1997 (1997-10-16) the whole document	1-50
A	CRUM C ET AL: "Complementary oligodeoxynucleotide mediated inhibition of tobacco mosaic virus RNA translation in vitro." NUCLEIC ACIDS RESEARCH, (1988 MAY 25) 16 (10) 4569-81., XP002168312	

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WEISS B ET AL: "Antisense RNA gene therapy for studying and modulating biological processes." CMLS CELLULAR AND MOLECULAR LIFE SCIENCES, vol. 55, no. 3, March 1999 (1999-03), pages 334-358, XP002168313 ISSN: 1420-682X cited in the application	
P,X	SCHULTE JANEL ET AL: "Mapping of antisense inhibition sites in the leader region of brome mosaic virus RNA 3." TEXAS JOURNAL OF SCIENCE, vol. 52, no. 3, August 2000 (2000-08), pages 213-222, XP001002356 ISSN: 0040-4403 figure 3	1,2,4,5, 11,15, 16,18, 20,21, 23-26,37

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ional Application No
PCT/US 01/00754

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0331939 A	13-09-1989	US 5580761 A	03-12-1996
		US 5324643 A	28-06-1994
US 5399676 A	21-03-1995	AU 641219 B	16-09-1993
		AU 7148191 A	31-05-1991
		CA 2071536 A	24-04-1991
		EP 0539371 A	05-05-1993
		JP 5505101 T	05-08-1993
		WO 9106626 A	16-05-1991
		US 5527899 A	18-06-1996
		US 5721218 A	24-02-1998
WO 9738097 A	16-10-1997	AU 5441896 A	30-10-1996
		WO 9632474 A	17-10-1996
		AU 2723497 A	29-10-1997